IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re United States Patent Application of:		**Customer No.:**	**23448**
Applicant:	**Von Knebel-Doeberitz, et al.**	**Docket No.:**	**4121-121**
Serial No.:	**09/719,336**	**Examiner:**	**C. Qian**
Date Filed:	**March 22, 2001**	**Art Group:**	**1633** #16
Title:	**USE OF ADENO-ASSOCIATED VIRUSES FOR DECREASING THE RADIOTHERAPY-INDUCED OR CHEMOTHERAPY-INDUCED RESISTANCE IN CANCER PATIENTS**	**Confirmation No.:**	**7154**

EXPRESS MAIL CERTIFICATE

I hereby certify that I am mailing the attached documents to the Commissioner for Patents on the date specified below, in an Express Mail envelope addressed to the Commissioner for Patents, P. O. Box 1450, Alexandria, VA 22313-1450 and Express Mailed under the provisions of 37 CFR 1.10.

Candace White

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SUPPLEMENTAL DECLARATION UNDER 37 C.F.R. 1.132 OF DR. MAGNUS VON KNEBEL-DOEBERITZ IN U.S. PATENT APPLICATION NO. 09/719,336

Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Magnus Von Knebel-Doeberitz, hereby declare:

1. THAT I am a named co-inventor of the invention that is described and claimed in U.S. Patent Application No. 09/719,336 filed in the United States Patent and Trademark Office on March 22, 2001 in the names of Magnus Von Knebel-Doeberitz, Petra Klein-Bauernschmitt, Harald Zur Hausen and Jorg Schlehofer for "USE OF ADENO-ASSOCIATED VIRUSES FOR DECREASING THE RADIOTHERAPY-INDUCED OR CHEMOTHERAPY-INDUCED RESISTANCE IN CANCER PATIENTS" (the "Application").

2. THAT the invention of the Application relates to the use of adeno-associated viruses for decreasing the radiotherapy-or chemotherapy-resistance in patients who suffer from a cancer that may be treated by radiotherapy or chemotherapy.

3. THAT a declaration was submitted on December 13, 2002, which discussed and showed that AAV-2 has tumor-suppressive activity and can sensitize freshly explanted human tumor tissues to γ irradiation and to various chemotherapeutic agents *in vivo*. Further, we showed that rats infected with the AAV-2 virus and treated with chemotherapeutic drugs remained in better physical condition compared to controls treated with only chemotherapy. Also, we showed that infection with AAV-2 enhanced the cytotoxic effect of 5-FU on pancreatic tumor cells and reduced tumor growth in immunocompetent Lewis rats that were challenged with syngeneic pancreatic cancer cells. Furthermore, we showed that chemotherapy-related toxic side effects, such as thrombocytopenia, neutropenia, loss of weight and pain, were significantly reduced in animals treated with concomitant AAV infection.

4. THAT I am familiar with the January 28, 2003 Office Action and the statement by the Examiner in the Office Action stating, "Applicants do not disclose whether these tumor cells are resistant to chemotherapy and radiotherapy."

5. THAT in response we expressly stated in the "Methods and Material" of the December 13, 2002 Declaration that we used the murine tumor cell line DSL6A which was originally derived from a primary **pancreatic** carcinoma of an azaserine-treated Lewis rat for the *in vivo* testing procedures. DSL6A tumor cells were injected s.c. into the abdominal flank of 4-week-old rats (10^6 cells in 100 µl PBS/animal).

6. THAT we used the cell line DSL6A because the cells are primary **pancreatic** carcinoma cells and it has been known by those skilled in the art for at least the last two decades that drug therapy has limited success in pancreatic cancer cells because pancreatic cancer cells are notoriously resistant to even the most powerful chemotherapy. To provide proof of this statement, we have included in Appendix B documentary evidence that substantiates the fact that it was known to those skilled in the art, at the time of filing, that pancreatic carcinoma cells were considered intrinsically drug resistant.

7. THAT the substantiating evidence includes:
   i) Vredevoe, et al. (1981) "Concepts of Oncology Nursing," page 86, Table 5.1 is included that expressly states that pancreatic cancers are poorly responsive to cancer treatments with chemotherapy;

ii) Goldstein, et al., (1989) *J. of Natl. Cancer Inst.* 81: 116-124, states that pancreas cancer tumors are included with intrinsically drug-resistant tumors (see abstract and page 121, column 1);

ii) U.S. Patent No.: 5,436,243 (1995) states that it is known that tumor cells derived from the pancreas are considered to be intrinsically drug resistant and do not respond to chemotherapy. (see column 3, lines 32-40, column 11, lines 63-66); and

iv) Iison, David H., (1997) Primary Care and Cancer, Vol. 17, No. 7, states that the well-known resistance of pancreatic cancer to chemotherapy prompts many patients to opt only for best supportive medical care without even a trial of chemotherapy.

8. Taken together the above-discussed references provide sufficient evidence that pancreatic cancer cells were known to be notoriously drug resistance at the time of filing the presently claimed invention and that using pancreatic cells in the testing procedures provided a definitive statement regarding the effectiveness of the claimed combination therapy. Thus, we specifically implanted the DSL6A cell line in the immunocompetent Lewis rat to determine if the inherent and well-known drug resistance of the pancreatic carcinoma cells was reversible with the AAV-2 infection in combination with chemotherapeutic agents.

9. THAT the above-recited effective results provide evidence that the drug resistance of the pancreatic cancer cells was reduced, if not completely reversed. Further, our findings confirm the potential use of AAV-2-mediated sensitization of tumor cells to cytotoxic drugs and demonstrate a clear prevention of chemotherapy-related toxic side effects. Additionally, in immunocompetent rats, combination of intratumoral AAV-2 infection of implanted pancreatic DSL6A tumors with additional systemic 5-FU chemotherapy resulted in significant retardation of tumor growth and prolonged survival, in which the combined effects clearly exceeded those achieved by administration of either agent alone.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statement may jeopardize the validity of the application or any patent issued thereon.

Magnus Von Knebel-Doeberitz, Ph.D.

APPENDIX B

PRENTICE-HALL, INC. *Englewood Cliffs, New Jersey 07632*

Concepts of ONCOLOGY NURSING

DONNA L. VREDEVOE, Ph.D.
Professor
Teaching in Graduate Nursing Oncology Program and Nursing Research
School of Nursing, UCLA

ANAYIS DERDIARIAN, R.N., M.N.
LINDA PATTI SARNA, R.N., M.N.
MAIRE FRIEL, R.N., M.N.
Assistant Clinical Professors
Teaching in Graduate Nursing Oncology Program
School of Nursing, UCLA

JOSEPHINE A. GATAN SHIPLACOFF, R.N., M.N.
Clinical Research Nurse Coordinator
Division of Surgical Oncology
Department of Surgery, UCLA

with contribution by

CAROL A. BRAINERD, R.N., M.N.
Oncology Program Director
Visiting Nurses Association of Pasadena and San Gabriel Valley
Pasadena, California

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TABLE 5.1
Cancer Treatment with Chemotherapy: Curability and Sensitivity [16]

Potentially Curative (highly responsive)
Acute lymphoblastic leukemia (childhood)
Hodgkin's disease
Gestational choriocarcinoma
Testicular cancer
Burkitt's lymphoma
Wilms's tumor
Long-Term Palliation (moderately responsive)
Carcinoma of the breast
Selected lymphomas
Multiple myeloma
Ovarian cancer
Prostate carcinoma
Chronic leukemia
Neuroblastoma
Small-cell carcinoma of the lung
Osteogenic sarcoma
Ewing's sarcoma
Soft-tissue sarcoma
Short-Term Palliation (fairly responsive)
Lung cancer (non-small cell)
Head and neck cancer (squamous)
Colon cancer
Liver cancer
Stomach cancer
Bladder cancer
Cervical cancer
Brain tumors
Gastric carcinoma
Occasional Palliation (poorly responsive)
Melanoma
Pancreatic cancer
Renal cell carcinoma

Note: Results are dependent on individual patient and tumor factors.

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ARTICLES

Expression of a Multidrug Resistance Gene in Human Cancers

Lori J. Goldstein, Hanan Galski, Antonio Fojo, Mark Willingham, Shinn-Liang Lai, Adi Gazdar, Robert Pirker, Alexander Green, William Crist, Garrett M. Brodeur, Michael Lieber, Jeffrey Cossman, Michael M. Gottesman, Ira Pastan*

Many cancers have been cured by chemotherapeutic agents. However, other cancers are intrinsically drug resistant, and some acquire resistance following chemotherapy. Cloning of the cDNA for the human MDR1 gene (also known as PGY1), which encodes the multidrug efflux protein P-glycoprotein, has made it possible to measure levels of MDR1 RNA in human cancers. We report the levels of MDR1 RNA in >400 human cancers. MDR1 RNA levels were usually elevated in untreated, intrinsically drug-resistant tumors, including those derived from the colon, kidney, adrenal gland, liver, and pancreas, as well as in carcinoid tumors, chronic myelogenous leukemia in blast crisis, and cell lines of non-small cell carcinoma of the lung (NSCLC) with neuroendocrine properties. MDR1 RNA levels were occasionally elevated in other untreated cancers, including neuroblastoma, acute lymphocytic leukemia (ALL) in adults, acute nonlymphocytic leukemia (ANLL) in adults, and indolent non-Hodgkin's lymphoma. MDR1 RNA levels were also increased in some cancers at relapse after chemotherapy, including ALL, ANLL, breast cancer, neuroblastoma, pheochromocytoma, and nodular, poorly differentiated lymphoma. Many types of drug-sensitive and drug-resistant tumors, including NSCLC and melanoma, contained undetectable or low levels of MDR1 RNA. The consistent association of MDR1 expression with several intrinsically resistant cancers and the increased expression of the MDR1 gene in certain cancers with acquired drug resistance indicate that the MDR1 gene contributes to multidrug resistance in many human cancers. Thus, evaluation of MDR1 gene expression may prove to be a valuable tool in the identification of individuals whose cancers are resistant to specific agents. The information may be useful in designing or altering chemotherapeutic protocols in these patients. [J Natl Cancer Inst 1989;81:116–124]

Chemotherapeutic agents have proven to be effective in the cure or palliation of some human cancers; however, both intrinsic drug resistance and acquired drug resistance remain clinical obstacles in the treatment of many other cancers. For the study of the mechanisms of multidrug resistance, tumor cell lines have been selected for resistance to the *Vinca* alkaloids, doxorubicin, dactinomycin, and related natural products (*1–5*). Intracellular drug accumulation has been found to be decreased secondary to increased drug efflux in these cell lines (*2,6*). These multidrug-resistant cell lines usually contain an amplified gene, termed MDR1 (also known as PGY1) in the human, that is transcribed into a 4.5-kilobase mRNA (*7–12*). The protein product of this gene is a 170-kilodalton

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L. J. Goldstein, H. Galski, A. Fojo, M. Willingham, M. M. Gottesman, and I. Pastan (Laboratory of Molecular Biology, Division of Cancer Biology and Diagnosis), S.-L. Lai and A. Gazdar (NCI-Navy Medical Oncology Branch, Division of Cancer Treatment), and J. Cossman (Laboratory of Pathology, Division of Cancer Biology and Diagnosis), National Cancer Institute, Bethesda, MD.

R. Pirker; Second Medical Clinic, Garnisongasse 13, A-1090 Vienna, Austria.

A. Green and W. Crist, Department of Hematology–Oncology, St. Jude Children's Research Hospital, and Department of Pediatrics, University of Tennessee, Memphis, TN.

G. M. Brodeur, Department of Pediatrics, Washington University School of Medicine, St. Louis, MO.

M. Lieber, Mayo Clinic, Rochester, MN.

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**Correspondence to:* Dr. Michael M. Gottesman, Bldg. 37, Rm. 2E18, National Institutes of Health, Bethesda, MD 20892.

membrane glycoprotein, called P-glycoprotein or the multidrug transporter, which is an energy-dependent drug efflux pump (*13,14*).

A full-length cDNA for the MDR1 gene from one of the multidrug-resistant human KB carcinoma cell lines has been isolated and sequenced (*15,16*). With the use of a region of this cDNA as a probe, the MDR1 gene has been shown to be expressed at a high level in normal human kidney, adrenal gland, liver, and colon (*17*). In the kidney, liver, and colon, the MDR1 gene product (P-glycoprotein) was present on the luminal surface of epithelial cells, which is consistent with a normal role of this protein as a transporter (*18*). In addition, several human cancers, including adenocarcinomas derived from tissues that normally express the MDR1 gene, have been shown to overexpress MDR1 RNA (*17,19*). Immunochemical analysis revealed overexpression of P-glycoprotein in two of five patients with ovarian carcinoma (*20*) and in two patients with drug-resistant acute nonlymphocytic leukemia (ANLL) (*21*). In 25 patients with sarcoma, six tumor samples had elevated levels of P-glycoprotein (*22*).

To investigate further the association of the expression of the MDR1 gene in human cancers with drug resistance, we have measured MDR1 RNA levels in many types of human cancers. We report here measurements of MDR1 RNA levels in >400 human cancer specimens. Our results identify four groups of cancers: (*a*) cancers that usually express high levels of MDR1 RNA, (*b*) cancers that occasionally express high levels, (*c*) cancers that rarely express MDR1 RNA, and (*d*) cancers that express the MDR1 gene at elevated levels after exposure to chemotherapeutic agents. Taken together, these results are consistent with an important role for the MDR1 gene in clinical drug resistance and suggest that measurements of MDR1 RNA can be useful in the design of chemotherapeutic protocols for certain tumors.

Materials and Methods

Cell Lines

KB-3-1 is the drug-sensitive parental KB (HeLa) cell line. KB-8-5, which is four times as resistant to doxorubicin and six times as resistant to vinblastine, was derived in two steps from KB-3-1 by selection in colchicine (*4*). KB-8-5 has increased levels of MDR1 mRNA without gene amplification (*7*). Cell line KB-C1 was derived in two further steps from KB-8-5 and is 160 times more resistant to doxorubicin and 96 times more resistant to vinblastine than KB-3-1 is (*6*). It has amplified the MDR1 gene about 100-fold and expresses MDR1 mRNA at a very high level (*7*).

MDR1 Hybridization Probes

cDNA was prepared with the use of RNA from KB-C2.5 cells, which contain large amounts of MDR1 mRNA, and was inserted into the EcoRI site of bacteriophage λgt11 (*15*). Probe 5A, which encodes about one-third of the coding region of a full-length MDR1 cDNA, was labeled by nick translation before use in the RNA slot blot analyses (*15*). An MDR1 genomic fragment of 785 base pairs (bp) that was derived from PvuII-digested plasmid pMDR-P2 was used to make a riboprobe with SP6 polymerase for the RNase protection assays. This fragment contains the transcription-initiation sites of the downstream promoter and additional sequences 5′ to the downstream promoter (*23*). Deoxycytidine 5′-[α-^{32}P]triphosphate (3,000 Ci/mmol; Ci = 37 GBq) and uridine 5′-[α-^{32}P]tirphosphate (3,000 Ci/mmol) were from DuPont/NEN Products (Boston, MA). Promega Biotec (Madison, WI) was the source of the PGEM4 and the Riboprobe Gemini System II. The Amersham Corporation (Arlington Heights, IL) manufactured the nick-translation system.

RNA Extraction and Electrophoresis

All samples were stored frozen at −70 °C. Before RNA extraction, solid tumors were pulverized on a metal surface on a bed of dry ice. Buffy coats from leukemia samples or leukemia blast cells isolated by Ficoll–Hypaque gradient centrifugation and frozen in 10% dimethyl sulfoxide were thawed rapidly at 37 °C and centrifuged. For lung cancer and mesothelioma, cell lines were available for analysis. The lung cancer cell lines were established, grown, and characterized as previously described (*24–28*). Tissue culture dishes and flasks of cell lines were washed twice with phosphate-buffered saline without calcium and magnesium. Total cellular RNA was extracted by homogenization in guanidinium isothiocyanate followed by centrifugation over a cesium chloride cushion (*29*) or by acid–phenol extraction (*30*). The RNA was electrophoresed in 1% agarose–6% formaldehyde gels. One microgram of total RNA was loaded per lane. The ribosomal RNA appeared undegraded in almost all samples reported here. Samples with degraded RNA were not further analyzed.

Slot Blot Analysis

Nitrocellulose filters were presoaked in 10 × SSC (1 × SSC = 0.15 *M* NaCl/15 m*M* sodium citrate, pH 7). Serial dilutions of 10, 3, 1, and 0.3 μg of each sample of total RNA in 10 × SSC were applied to each well of a Schleicher and Schuell slot blot apparatus. After baking at 80 °C in a vacuum oven, the filters were prehybridized for 4–6 hours at 42 °C in 50% formamide, 5 × SSC, 5 × Denhardt's solution (1 × Denhart's solution = 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% acetylated bovine serum albumin), 50 m*M* sodium phosphate (pH 6.5), and 200 μg of salmon sperm DNA/mL. The filters were then hybridized for 16 hours at 42 °C in 50% formamide, 5 × SSC, 1 × Denhardt's solution, 10% dextran sulfate, 100 μg of salmon sperm DNA/mL, and 20 m*M* sodium phosphate (pH 6.5) with 5 × 10^6 cpm of nick-translated cDNA/mL. After hybridization, the filters were washed four times for a total of 1 hour with 1 × SSC/0.1% sodium dodecyl sulfate (SDS) at 23 °C followed by two 10-minute washes with 0.2× SSC/0.1% SDS at 50 °C. Autoradiographs were exposed for 1–5 days. Hybridization with a nick-translated γ-actin probe (*31*) was performed to compare RNA loading.

RNase Protection Assay

The starting sites of MDR1 transcription in various human cell lines and tumors were mapped with an RNase protection

assay with the use of a labeled SP6 anti-sense RNA probe (785 nucleotides) derived from the PvuII-digested plasmid described above. Twenty micrograms of total RNA from each sample was hybridized with 3×10^5 cpm of the riboprobe, and RNase digestion was performed as previously described (*23,32*).

Results

Quantitation of MDR1 RNA

MDR1 RNA was routinely measured by a slot blot procedure in which various amounts of RNA from unknown and known samples were applied to the same blot. A typical RNA slot blot is illustrated in figure 1. RNA from KB-3-1 cells, which are drug sensitive, and RNA from KB-8-5 cells, which are about fivefold multidrug resistant, were included in each blot. Relative to KB-3-1 cells, the KB-8-5 cells have a 30- to 40-fold increase in MDR1 mRNA (*17*). On this basis, the signal intensity of 10 μg of KB-8-5 total RNA was assigned an arbitrary value of 30 U. The value of the signal from each tumor is expressed relative to that of the signal from KB-8-5 RNA. KB-8-5 RNA gives a reproducible and easily detectable signal. To ensure reproducibility of results, we normalized the quantity of RNA loaded for the amount of actin RNA detected. Normalization was usually not necessary, since the amount of RNA was similar in all the blots (fig. 1).

Cancers With High Levels of MDR1 RNA

MDR1 expression was considered to be high if ≥50% of the cancers in each group had detectable levels of MDR1 RNA. In a substantial proportion of the cancers, MDR1 RNA levels were ≥30 U (table 1). Levels of MDR1 RNA were high in several types of untreated cancers, including colon cancer, renal cell carcinoma, hepatoma, adrenocortical carcinoma, pheochromocytoma, islet cell tumor of the pancreas, chronic myelogenous leukemia (CML) in blast crisis, and carcinoid tumor, as well as in cell lines of non-small cell lung cancer with neuroendocrine properties (NSCLC-NE). Typical results from colon and adrenocortical carcinomas are shown in figure 1. The range of signals in four carcinoid tumors is illustrated by the RNA analysis in figure 2.

We performed RNase protection experiments to determine whether MDR1 RNA in these specimens that contained elevated RNA levels initiated only at the downstream promoter used by normal human tissues or also at an upstream promoter detected in some multidrug-resistant cell lines. RNA preparations from most colon carcinomas and adrenocortical cancers and some carcinoid tumors, leukemias, and pheochromocytomas containing ≥30 U of MDR1 RNA were used for these analyses (fig. 3). For these analyses, a 785-bp RNA, representing genomic sequences encompassing the promoter region and >100 bp of the 5′ region of the MDR1 mRNA, was hybridized with the RNA samples in solution and then digested with RNase. Two fragments were detected when RNA from KB-8-5 cells and RNA from KB-C1 cells were analyzed, corresponding to two major transcription-initiation sites. The two fragments of 323 and 130 bp, respectively, are indicated by arrows on figure 3 and correspond to mRNA initiated at the upstream and the downstream promoters. In the specimens listed above from patients who had not previously received chemotherapy, only initiation from the downstream site was detected. The amounts of MDR1 RNA detected by RNase protection were similar to those detected by the slot blot analyses, which validates the use of slot blots for detecting MDR1 RNA levels.

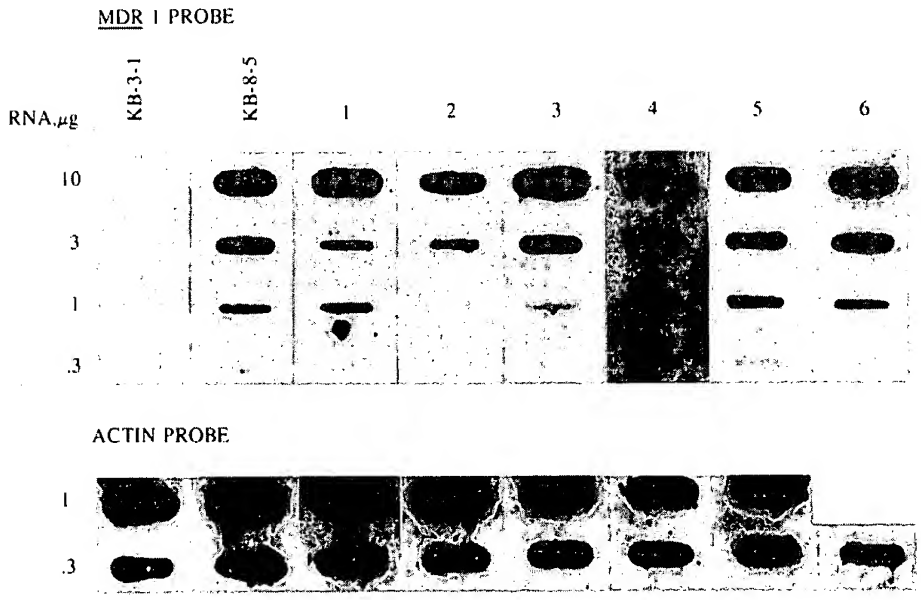

Figure 1. Slot blot analysis of MDR1 expression in untreated human cancers. Lanes 1–3: total RNA samples from colon cancer specimens. Lanes 4–6: RNA samples from adrenocortical carcinomas. Serial dilutions of 10, 3, 1, and 0.3 μg of total RNA were applied to each well. Hybridization of blot with γ-actin probe demonstrated comparable amounts of RNA loaded in all wells. KB-3-1 = drug-sensitive parental KB cell line; KB-8-5 = multidrug-resistant KB subline.

Table 1. Generally high MDR1 RNA levels in untreated cancers*

Cancer type/cell line	Total No. of cancers	No. positive (≥30 U)	No. low positive (2–29 U)	% positive	Reference
Colon carcinoma	41	10	25	85	†,*17*
Renal cell carcinoma	50	35	5	80	†,*19*
Hepatoma	12	7	5	100	†
Adrenocortical cancer	9	6	1	77	†,*17*
Pheochromocytoma	20	11	4	75	†,*17*
Islet cell tumor of pancreas	4	2	0	50	†
CML (blast crisis)	3	3	0	100	†
Carcinoid tumor	9	2	5	77	†
NSCLC-NE (cell lines)	6	2	3	83	†

*MDR1 RNA levels were measured by RNA slot blot analysis and are expressed relative to the level in the drug-resistant KB-8-5 cell line, which has been assigned a value of 30 U for the expression of 10 μg total RNA.

†This work.

‡Lai S-L, Goldstein LJ, Gottesman MM, et al: detailed analysis in preparation.

Cancers With Intermediate Levels of MDR1 RNA

Some untreated cancers were found to have detectable levels of MDR1 RNA ≤50% of the time. Included in this group were adult acute lymphocytic leukemia (ALL), adult ANLL, non-Hodgkin's lymphoma, and neuroblastoma (table 2).

Cancers With Low or Undetectable Levels of MDR1 RNA

A large variety of untreated cancers were found to have generally low (<30 U) or undetectable levels of MDR1 RNA. These cancers included breast cancer, non-small cell lung cancer (NSCLC), bladder cancer, CML in chronic phase, esophageal carcinoma, gastric carcinoma, head and neck cancer, melanoma, mesothelioma, ovarian carcinoma, prostate cancer, sarcoma, small cell lung cancer (SCLC), thymoma, thyroid cancer, and Wilms' tumor (table 3). For nine specimens of squamous cell carcinoma of the lung (included in NSCLC), adjacent normal lung and tumor tissues from each patient were evaluated for expression, and no significant difference in MDR1 RNA expression was found (data not shown).

Figure 4 illustrates the distribution of MDR1 RNA expression in a few representative untreated cancers. Because of the wide range of RNA expression detected, a log scale was used. In this graph it is evident that most of the specimens of adrenocortical cancer and colorectal cancer had relatively high levels of MDR1 RNA, whereas most of the breast cancer specimens and most of the Wilms' tumor specimens had undetectable MDR1 RNA levels, with only a few samples having low MDR1 RNA levels.

Levels of MDR1 RNA in Relapsed Cancers

Cancers that were initially sensitive to chemotherapy but that relapsed after treatment were also examined. Table 4 lists those cancers in which we found high levels of MDR1 RNA after treatment with chemotherapy. These cancers included non-Hodgkin's lymphoma, neuroblastoma, pheochromocytoma, breast cancer, CML in blast crisis, adult ALL, and adult ANLL. In most cases we were not able to obtain specimens from the same patient before and after treatment. However, we did obtain such specimens from one child with ALL (Rothenberg M, Mickley L, Cole D, et al.: manuscript submitted for publication), from one patient with pheochromocytoma, and from two patients with non-Hodgkin's lymphoma. One of the two patients with non-Hodgkin's lymphoma had an MDR1 RNA level of 8 prior to chemotherapy. This patient was then treated with ProMACE–MOPP chemotherapy (cyclophosphamide, doxorubicin, etoposide, prednisone, mechlorethamine, vincristine, and procarbazine). At disease relapse, the MDR1 RNA level increased to 24. This tumor

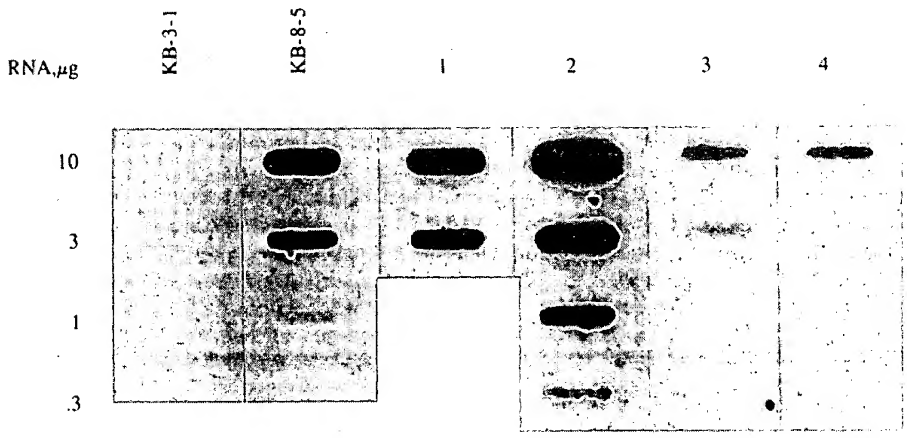

Figure 2. MDR1 expression in carcinoid tumors. Slot blot analysis from four different untreated carcinoid tumors. Serial dilutions of 10, 3, 1, and 0.3 μg of total RNA from each tumor were applied to each well. Hybridization of blot with γ-actin probe demonstrated comparable amounts of RNA loaded in all wells (data not shown). KB-3-1 = drug-sensitive parental KB cell line; KB-8-5 = multidrug-resistant KB subline.

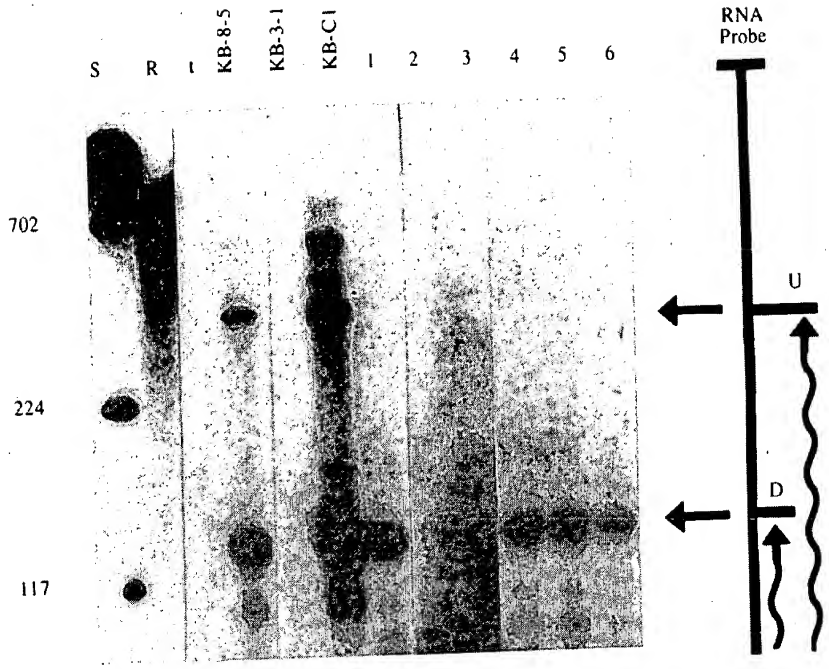

Figure 3. RNase protection assay of untreated cancers with elevated MDR1 RNA levels by slot blot analysis. Samples 1–6 are the same as in fig. 1. In each assay 20 μg of total RNA was used. Two bands were identified when RNA from KB-8-5 cell line and RNA from KB-C1 cell line were used, corresponding to two major initiation sites (designated "U" and "D" for upstream and downstream promoters, respectively). Only the band arising from downstream initiation site is present in these cancers. KB-3-1 = drug-sensitive parental KB cell line; KB-8-5 and KB-C1 = multidrug-resistant KB sublines; S = molecular weight standard; R = riboprobe; t = tRNA.

Table 2. Occasionally high MDR1 RNA levels in untreated cancers

Cancer type	Total No. of cancers	No. positive (≥30 U)	No. low positive (2–29 U)	% positive	Reference
All (adult)	15	2	0	13	*
ANLL (adult)	24	3	0	13	*
Non-Hodgkin's lymphoma	18	1	3	22	*
Neuroblastoma	34	1	16	50	†

*This work.
†Goldstein LJ, Fojo A, Gottesman MM, et al: detailed analysis in preparation.

Table 3. Low MDR1 RNA levels in untreated cancers

Cancer type/cell line	Total No. of cancers	No. positive (≥30 U)	No. low positive (2–29 U)	% positive	Reference
Breast cancer	57	0	9	15	*
NSCLC					
Tissue	19	0	7	36	†
Cell lines	30	0	5	16	†
Bladder cancer	6	0	1	16	*
CML (chronic phase)	3	0	0	0	*
Esophageal carcinoma	14	0	0	0	*
Gastric carcinoma	2	0	0	0	*
Head and neck cancer	14	0	0	0	*
Melanoma	3	0	0	0	*
Mesothelioma (cell lines)	20	0	1	5	*
Ovarian carcinoma	16	0	0	0	*
Prostate cancer	3	0	0	0	*
Sarcoma	11	0	0	0	*
SCLC (cell lines)	21‡	0	0	0	†
Thymoma	1	0	0	0	*
Thyroid cancer	4	0	0	0	*
Wilms' tumor	20	0	0	0	*

*This work.
†Lai S-L, Goldstein LJ, Gottesman MM, et al.: detailed analysis in preparation.
‡One sample was tumor tissue.

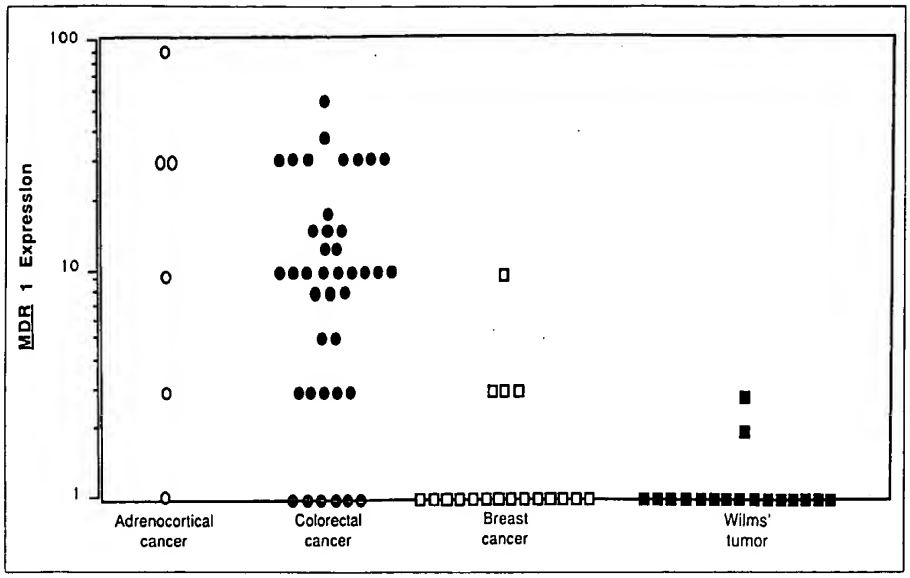

Figure 4. Quantitation of MDR1 expression in representative untreated cancers. Results obtained in slot blot analysis are graphically displayed for adrenocortical cancer, colorectal cancer, breast cancer, and Wilms' tumor. Values of individual tumors were expressed relative to the expression of the multidrug-resistant KB-8-5 cell line, which was arbitrarily assigned a value of 30 U for intensity of 10 μg of total RNA and gave an easily detectable and reproducible signal.

was a nodular, poorly differentiated lymphoma. The other lymphoma specimen studied was also of an indolent histology.

Discussion

Common Expression of MDR1 Gene in Cancers

In this study using RNA slot blot analysis, we have measured the expression of the MDR1 gene in >400 human cancers. Our results show that slot blot analysis is a sensitive method for quantitation of the MDR1 gene expression in human tumors and that many human tumors express MDR1 RNA. We have identified a group of untreated cancers that usually have elevated levels of MDR1 RNA. This group includes colon cancer, adrenocortical cancer, pheochromocytoma, hepatoma, pancreatic carcinoma, and renal cell carcinoma. All of these cancers are derived from tissues that normally have relatively high levels of MDR1 RNA. These findings confirm that the MDR1 gene can continue to be expressed when a normal cell undergoes malignant transformation. All of these cancers are known to be clinically resistant to chemotherapy, and thus the MDR1 gene may be implicated in their intrinsic drug resistance.

Variability in MDR1 Expression

Within the group of cancers with high MDR1 RNA levels, we observed considerable variation from cancer to cancer (fig. 4). For example, the highest MDR1 RNA level in colon cancer was 60 and the lowest was 0; the highest adrenal cancer MDR1 RNA level was 90 and the lowest was 0. This variation was not a technical artifact due to the quality of RNA because all RNA samples were checked for intactness of the RNA by gels and for quantity by analysis of actin RNA levels. However, a number of other factors need further examination. One is the number of cancer cells and stromal cells in each specimen. Stromal cells such as fibroblasts and inflammatory cells tend to have very low MDR1 RNA levels. A second factor is the state of differentiation of the cancer. We have observed in kidney cancers (*19*) and colon cancers (Fojo A: unpublished data) that MDR1 RNA levels tend to be lower in less differentiated cancers. A third factor is the cell type from which the cancer emerges. For example, in the kidney, most cancers showed histological evidence of being derived from proximal tubules (*33*), and the MDR1 gene was preferentially expressed in proximal tubules. In the pancreas, the MDR1 gene was preferentially expressed in collecting ductules. Although we have examined only four pancreatic cancers, the variable expression in this cancer could reflect the origin of the tumor.

Within the various types of lung tumors that have been examined, only one group, NSCLC-NE, tended to have high MDR1 RNA levels. A detailed analysis of this group will be published elsewhere (Lai S-L, Goldstein LJ, Gottesman MM, et al.: manuscript in preparation). The group of untreated cancers that occasionally had high MDR1 RNA levels included ALL, ANLL, non-Hodgkin's lymphoma, and neuroblastoma. These cancers are usually sensitive to chemotherapy. It will be important to gather more data to determine if the occasionally elevated levels of MDR1 RNA are associated with the occasional treatment failures seen in these cancers.

Low or undetectable levels of MDR1 RNA were seen in many cancers, including some that are drug sensitive and several others that are generally considered to be resistant or poorly responsive to chemotherapy (e.g., lung cancers). Other mechanisms of drug resistance probably operate in these cancers, or heterogeneity of MDR1 RNA expression could account for resistant subpopulations in these cancers. In the case of breast cancer and NSCLC, some expression of MDR1 RNA was seen in 15%–36% of the tumors examined, which is consistent with the latter possibility. For breast cancer, in particular, in which most of the cells may be stromal,

Table 4. MDR1 RNA levels in turmors relapsing after treatment

Cancer type	Chemotherapy*	Total No. of cancers	No. positive (≥30 U)†	No. low positive (2–29 U)†	% positive	Reference
Non-Hodgin's lymphoma	−	18	1	3	22	‡
	+	5	1	2	60	
Neuroblastoma	−	34	1	16	50	§
	+	16	5	11	100	
Pheochromocytoma	−	20	11	4	75	‡,*17*
	+	1	1	0	100	
Breast cancer	−	57	0	9	15	‡
	+	2	0	2	100	
CML						
Chronic phase ¶	−	3	0	0	0	‡
Blast crisis	−	3	3	0	100	
Blast crisis	+	3	2	0	66	**
ALL (adult)	−	15	2	0	13	‡
	+	1	1	0	100	
ANLL (adult)	−	24	3	0	13	‡
	+	5	2	2	80	
ALL (childhood)	−	9‡‡	n	n	11	‡‡
	+	20‡‡	n	n	15	

* − = no chemotherapy; + =chemotherapy given.
† n = not evaluated by quantitative slot blot analysis.
‡ This work.
§ Goldstein LJ, Foho A, Gottesman MM, et al.: detailed analysis in preparation
¶ Samples from CML in chronic phase and CML in blast crisis with and without chemotherapy are from different patients.
** Pirker R, Goldstein LJ, Ludwig H: detailed analysis in preparation.
†† Samples analyzed by Northern blot and RNase protection only.
‡‡ Rothenberg M, Mickley L, Cold D, et al.: manuscript for publication.

a low level of MDR1 RNA expression could be significant. To investigate the existence of heterogeneous expression, immunohistochemical or in situ hybridization studies of tumor specimens may allow one to distinguish the differential expression of various cell subpopulations.

Acquired Drug Resistance

Several lines of evidence now exist that indicate expression of the MDR1 gene may be partly responsible for acquired clinical drug resistance. In addition to the data reported here showing increased MDR1 RNA levels in ALL, ANLL, lymphoma, breast cancer, pheochromocytoma, CML in blast crisis, and neuroblastoma, antibodies have been used to demonstrate significant levels of P-glycoprotein in some patients with treated ovarian carcinoma, sarcoma, and leukemia (*20–22*). Clearly, further analysis of pretreatment and posttreatment MDR1 RNA levels and/or P-glycoprotein levels in the same patient is needed to prove the association of increased MDR1 RNA levels with acquired drug resistance. In tumors with acquired drug resistance, the measurement of elevated MDR1 RNA levels may help direct further chemotherapy by suggesting that agents affected by the multidrug-resistance phenotype (i.e., *Vinca* alkaloids, anthracyclines, and epipodophyllotoxins) not be used and that alternative treatments be considered.

In addition to observing elevated MDR1 RNA levels in cancers that were intrinsically resistant or that had acquired resistance after treatment, we observed increased MDR1 RNA levels in three patients with CML who had undergone blast crisis. This result raises the possibility that some step that leads to cancer progression, perhaps oncogene activation, could also lead to expression of the MDR1 gene. It has been previously reported that MDR1 RNA levels are elevated in chemically induced tumors of the liver (*34*), a result consistent with simultaneous activation of an oncogene and MDR1 RNA expression.

Characterization of MDR1 RNA in Cancers

RNase protection assays of many cancers that had positive expression confirmed the expression data of slot blot analysis. This protection assay is more specific than the slot blot assay, since the protection assay does not detect RNA transcribed from the closely related MDR2 gene, which has not been associated with multidrug resistance (*12,35*). The RNase protection assay has also allowed us to determine that transcription of the MDR1 gene in cancers of the colon and adrenal gland and carcinoid tumors occurs from the downstream promoter, as does transcription in normal adrenal glands and colon tissues (*23*). Because some drug-resistant tissue culture cell lines also use an upstream promoter, we have continued analyzing cancers to determine which promoters are used. We have found that in the specimens from two of the four children with ALL with elevated MDR1 RNA levels reported here, transcription initiated at both the upstream and downstream promoters; in contrast, in the specimens from the other two children, only the upstream promoter was used (Rothenberg M, Fojo A: unpublished data). The use of two promoter sites has also been seen in both treated and un-

treated adult leukemias and lymphomas that have elevated levels of MDR1 RNA (Goldstein LJ, Pastan I, Gottesman MM: unpublished data). The use of an upstream promoter in drug-resistant tumors suggests a different mechanism of regulation of expression of the MDR1 gene in such instances.

Evidence Linking MDR1 Expression to Multidrug Resistance

Our results have shown that cancers which are clinically drug resistant generally have elevated MDR1 RNA levels. Several lines of evidence suggest that multidrug resistance in cancers with elevated MDR1 expression is due, at least in part, to this expression: (*a*) when full-length cDNAs for the human or mouse MDR1 gene are transfected (*36,37*) or infected into human cells (*38,39*), these cells become multidrug resistant; (*b*) unselected cell lines from tumors, such as renal adenocarcinoma with elevated MDR1 RNA levels, have a multidrug-resistant phenotype, and their resistance is reversible by use of verapamil and quinindine (*40*), which are inhibitors of the multidrug transporter (*14*); and (*c*) there is some correlation between MDR1 RNA levels in renal adenocarcinomas and resistance of tumor explants to vinblastine (*19*). Based on these results, controlled clinical trials in patients with colorectal and renal cancers are under way with the use of quinidine as a reversing agent in conjunction with cytotoxic therapy including doxorubicin, etoposide, and vinblastine. Another direction of further investigation will be to develop other less toxic reversing agents.

Conclusions

We have measured levels of MDR1 mRNA in many human cancers. We have found elevated expression of the MDR1 gene in certain untreated cancers and in some treated cancers. Although the absence of MDR1 RNA expression in some drug-resistant cancers suggests that other mechanisms of multidrug resistance exist, the widespread occurrence of MDR1 RNA expression in drug-resistant cancers suggests that the MDR1 gene plays an important clinical role in many cancers. We estimate ≈450,000 new cases of cancers expressing the MDR1 gene per year on the basis of our expression data and the incidence of these cancers. Prospective trials correlating measurements of MDR1 RNA expression with clinical response to therapy will determine if MDR1 levels are predictive of response. If they are, MDR1 RNA measurements may be useful in the design or the alteration of chemotherapeutic regimens in individual patients.

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United States Patent [19]

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[11] Patent Number: 5,436,243

[45] Date of Patent: Jul. 25, 1995

[54] **AMINOANTHRAQUINONE DERIVATIVES TO COMBAT MULTIDRUG RESISTANCE**

[75] Inventors: **Clifford W. Sachs; Robert L. Fine,** both of Durham; **Lawrence M Ballas,** Apex; **R. Ivy Carroll; Robert Bell,** both of Durham, all of N.C.

[73] Assignees: **Research Triangle Institute Duke University,** Research Triangle Park, N.C.; **Pharmaceuticals Corporation,** Durham, N.C.

[21] Appl. No.: **152,894**

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[51] Int. Cl.[6] **A61K 31/135;** A61K 31/55; A61K 31/535; A61K 31/445

[52] U.S. Cl. **514/231.8;** 514/237.5; 514/239.5; 514/252; 514/255; 514/316; 514/319; 514/325; 514/649; 544/79; 544/156; 544/357; 544/380; 546/191; 546/204; 552/247; 552/255

[58] **Field of Search** 544/79, 156, 357, 380; 546/191, 204; 552/247, 255; 514/231.8, 237.5, 239.5, 252, 255, 316, 319, 325, 649

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Primary Examiner—Richard L. Raymond
Attorney, Agent, or Firm—Banner & Allegretti, Ltd.

[57] **ABSTRACT**

Potentiating agents inhibit the development of multidrug resistance, reduce drug-resistance in drug-resistant tumors, or sensitize tumors to antineoplastic drugs, thereby potentiating the effect of antineoplastic agents. The potentiating agents are aminoanthraquinones, preferably 1,4-bis(N-substituted) amino anthraquinones, and pharmaceutically acceptable salts thereof.

20 Claims, 13 Drawing Sheets

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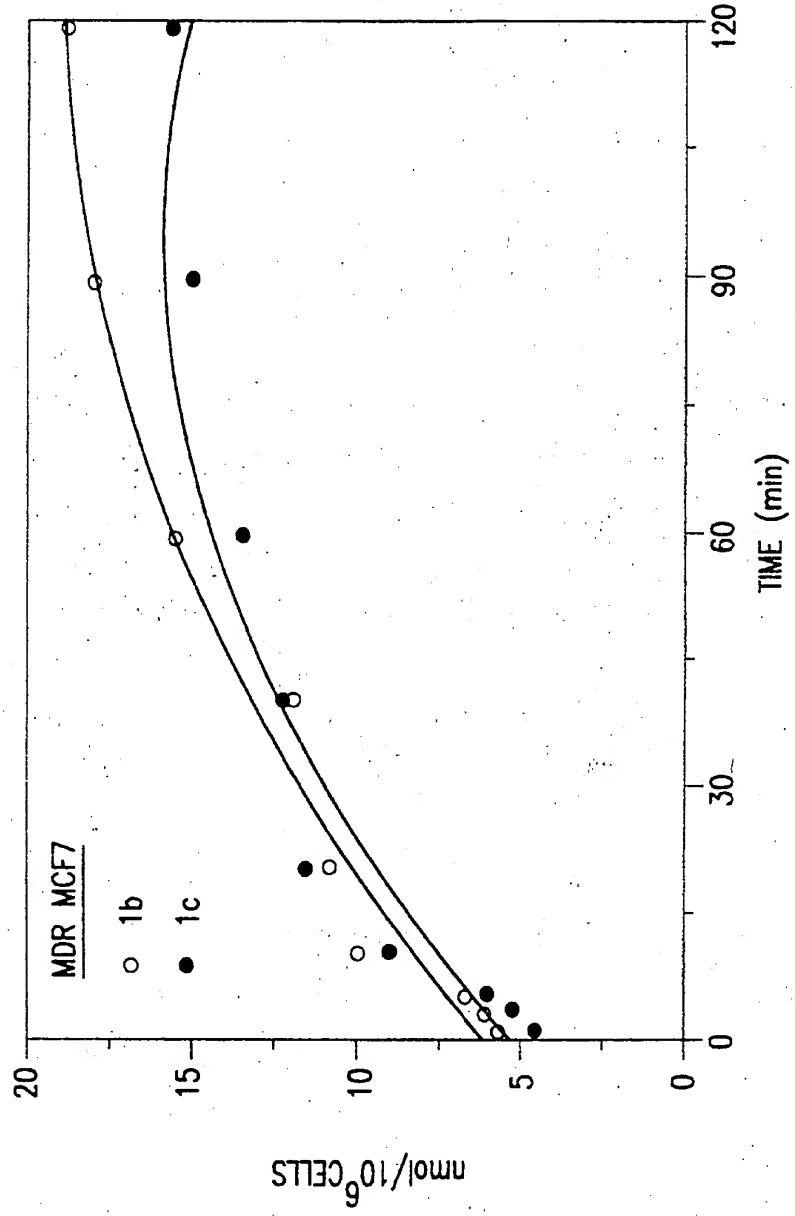


FIG. 1

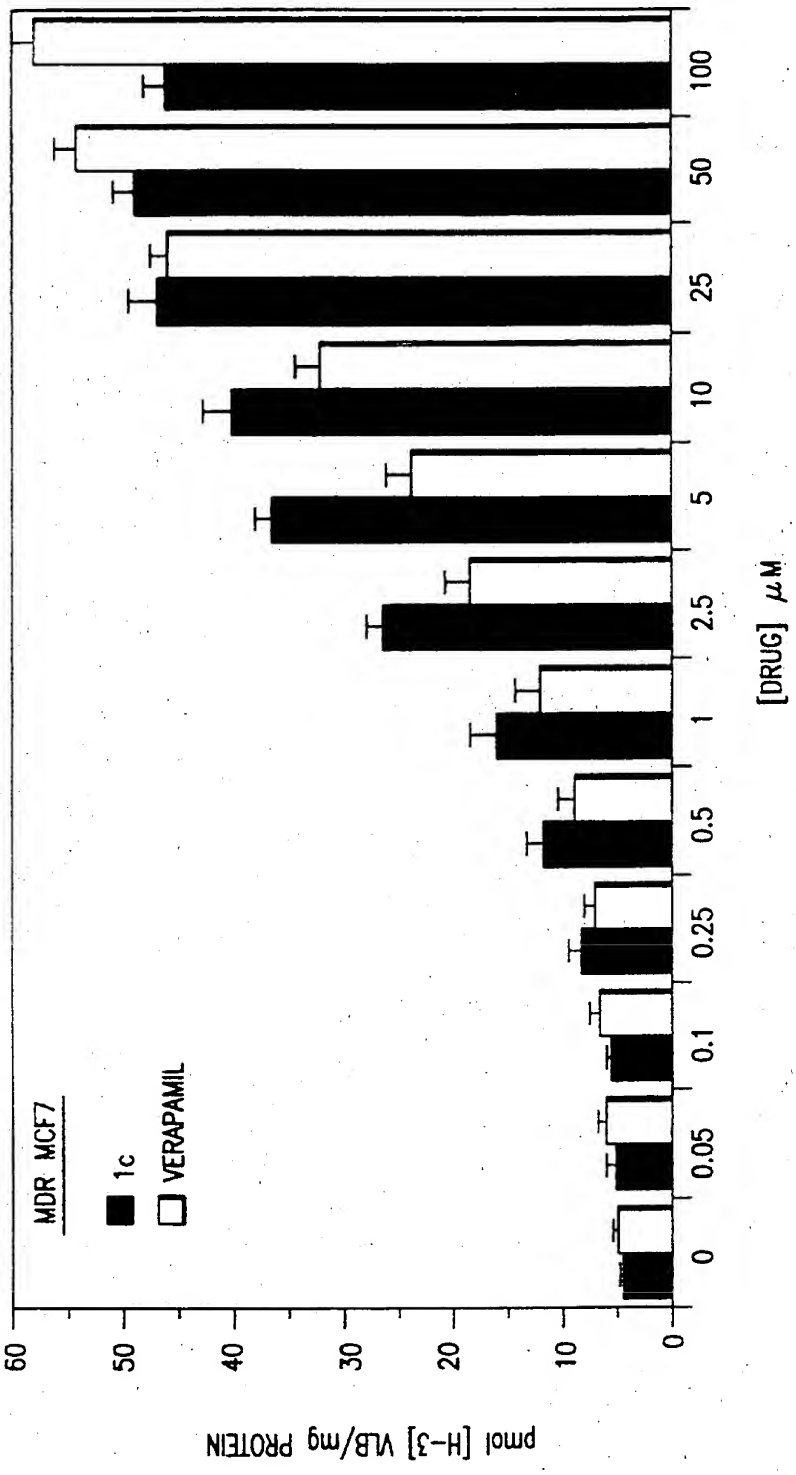

MDR MCF7
1c
VERAPAMIL
pmol [H-3] VLB/mg PROTEIN
0
10
20
30
40
50
60
0
0.05
0.1
0.25
0.5
1
2.5
5
10
25
50
100
[DRUG] μM

FIG.2

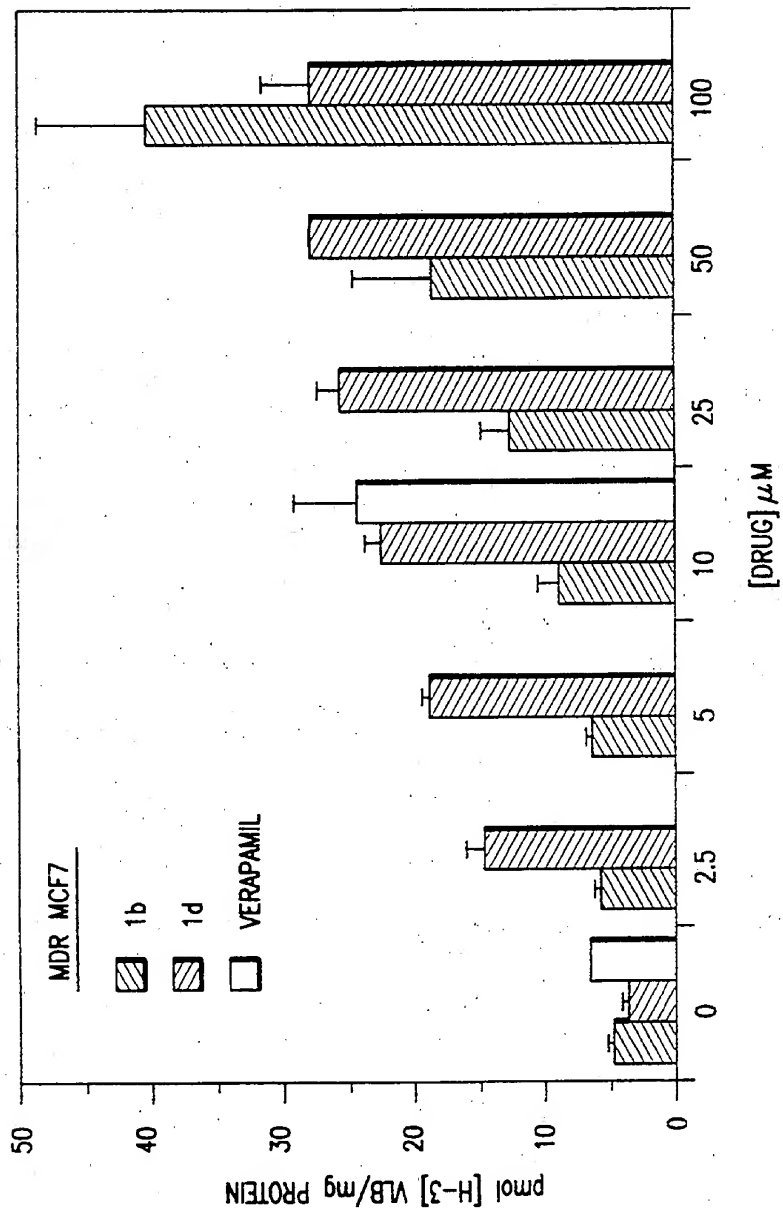

MDR MCF7
1b
1d
VERAPAMIL
pmol [H-3] VLB/mg PROTEIN
0
10
20
30
40
50
0
2.5
5
10
25
50
100
[DRUG] μM

FIG.3

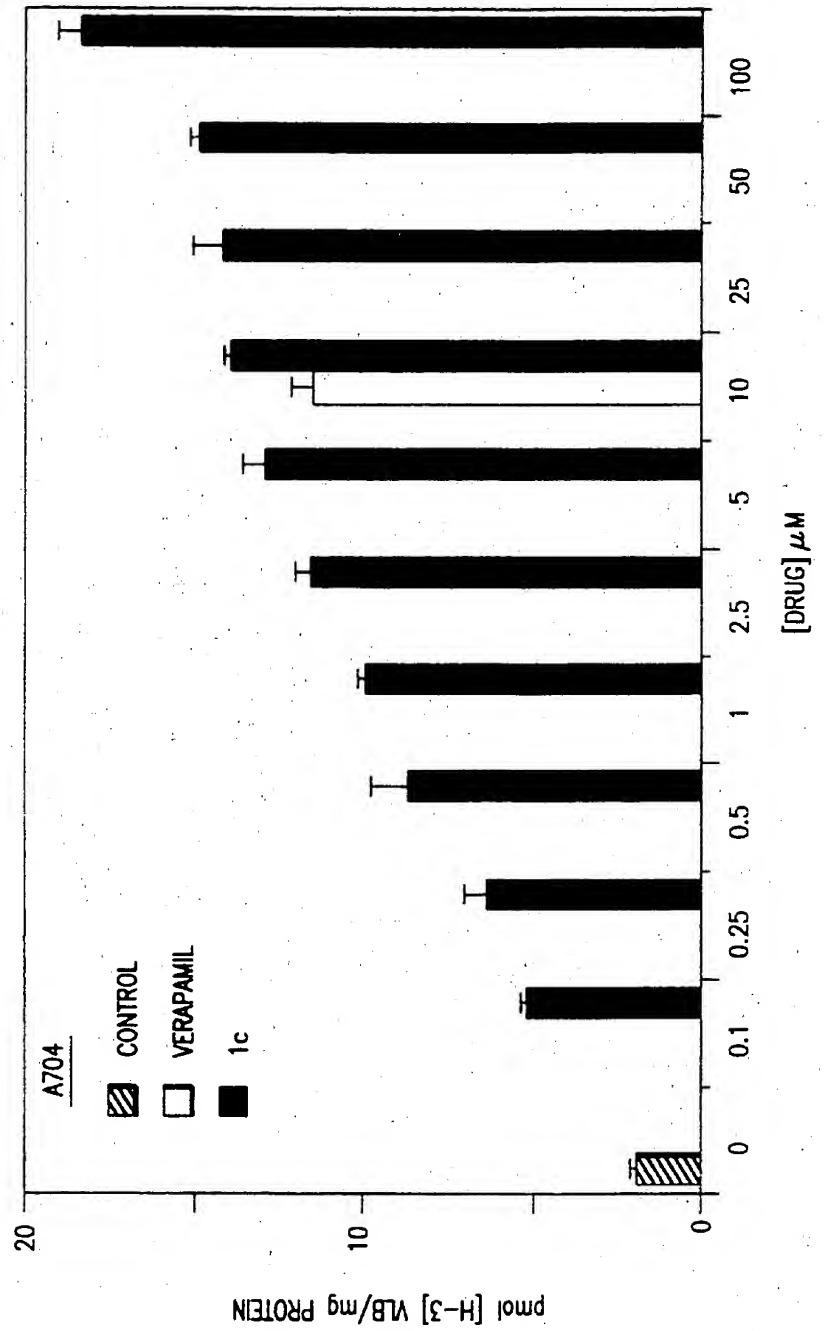

A704
CONTROL
VERAPAMIL
1c
pmol [H-3] VLB/mg PROTEIN
20
10
0
0
0.1
0.25
0.5
1
2.5
5
10
25
50
100
[DRUG] μM

FIG. 4

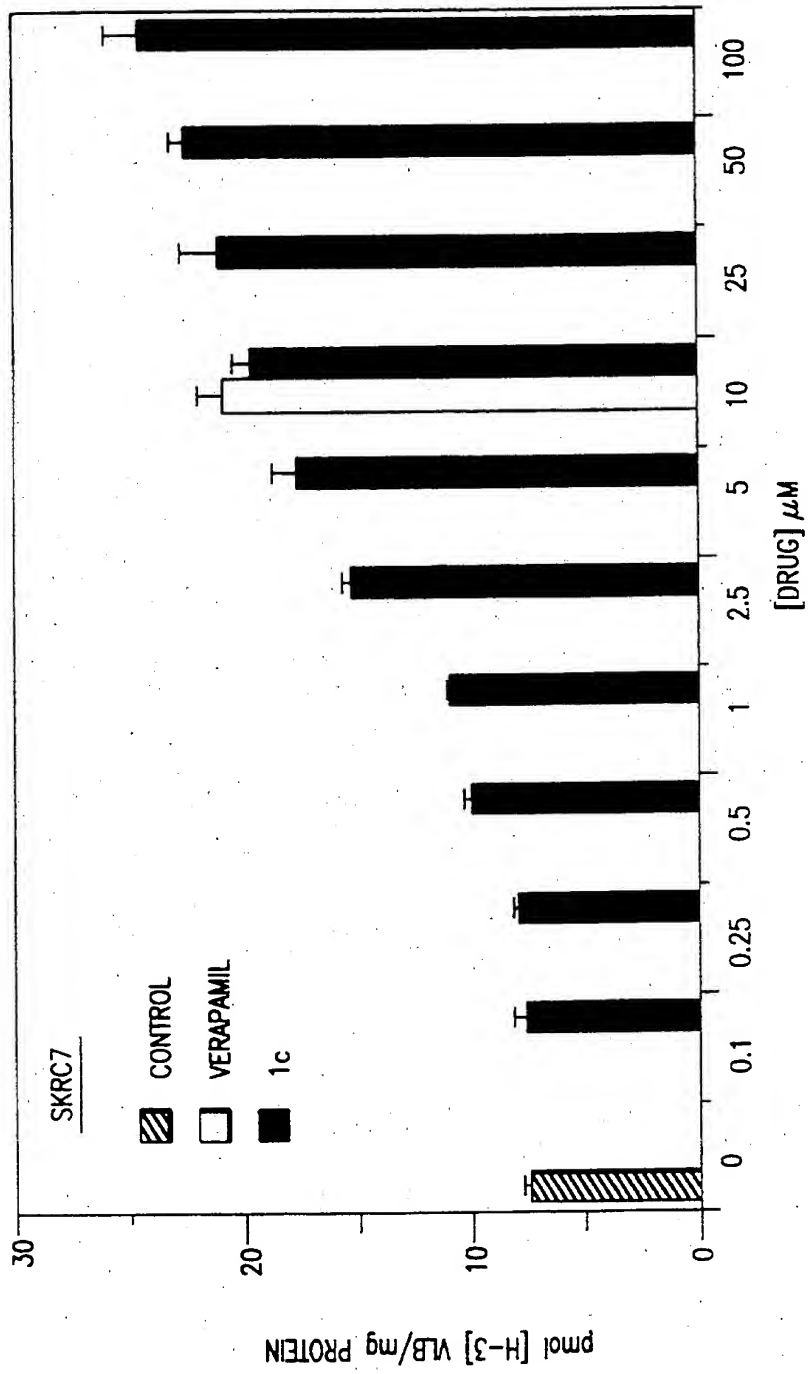

SKRC7
CONTROL
VERAPAMIL
1c
pmol [H-3] VLB/mg PROTEIN
0
10
20
30
0
0.1
0.25
0.5
1
2.5
5
10
25
50
100
[DRUG] μM

FIG.5

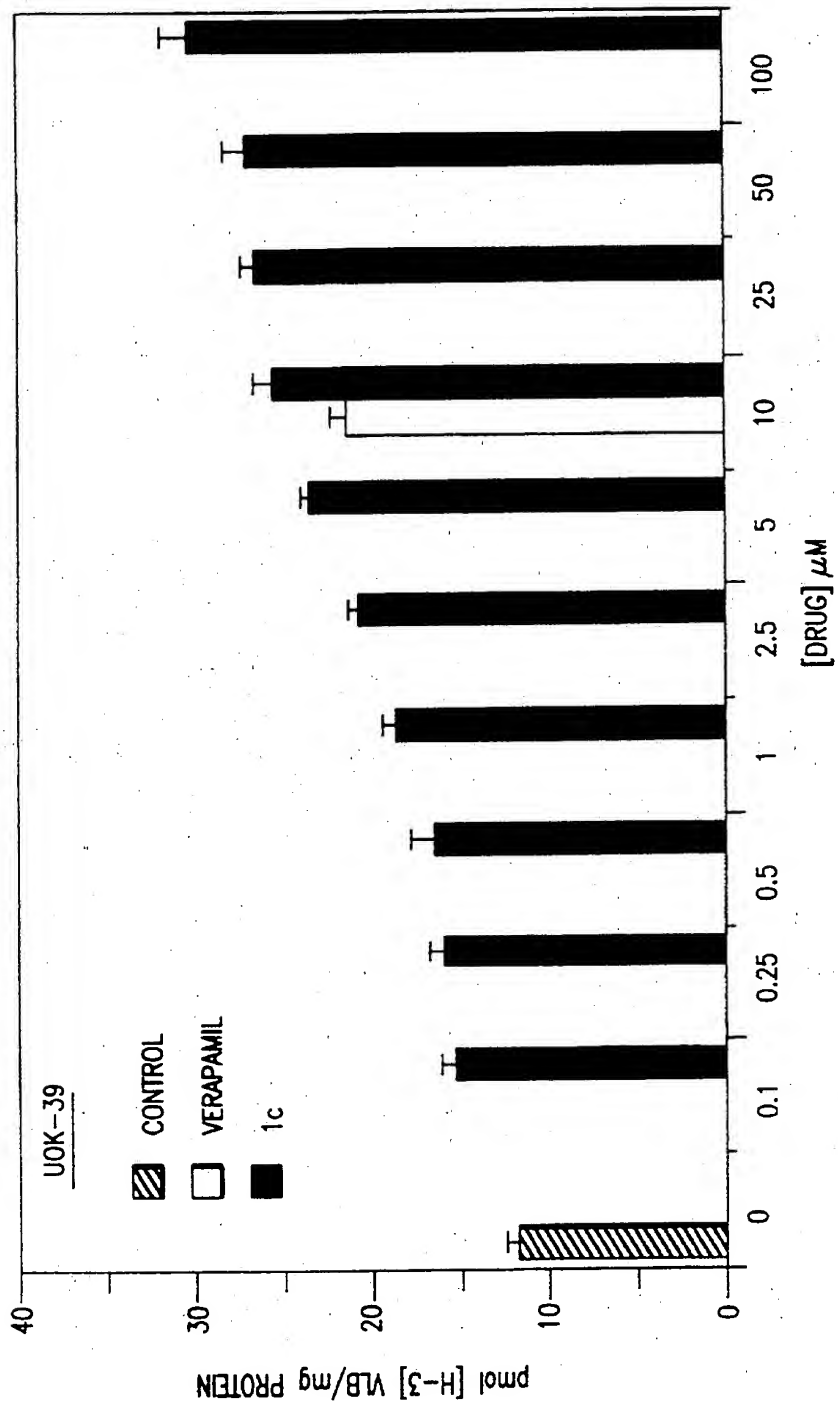

UOK-39
CONTROL
VERAPAMIL
1c
pmol [H-3] VLB/mg PROTEIN
0
10
20
30
40
0
0.1
0.25
0.5
1
2.5
5
10
25
50
100
[DRUG] μM

FIG.6

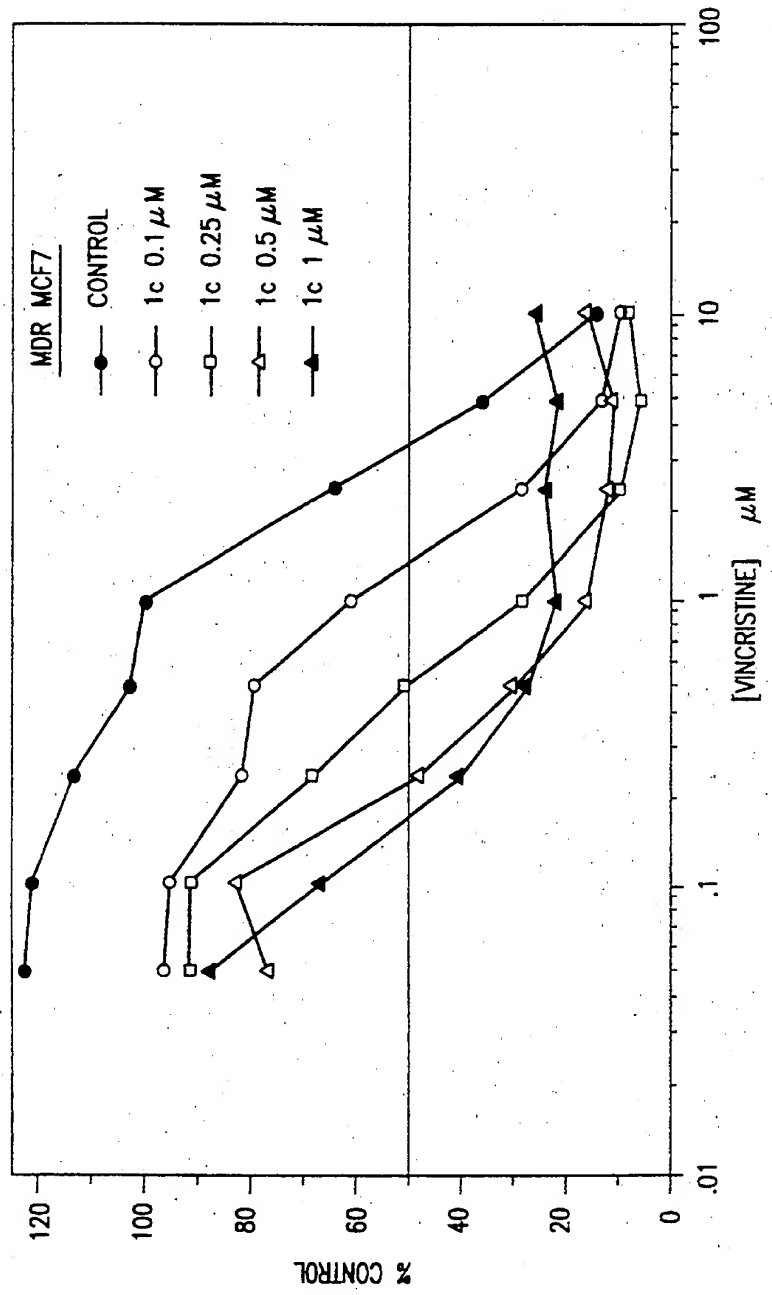

MDR MCF7
CONTROL
1c 0.1 μM
1c 0.25 μM
1c 0.5 μM
1c 1 μM
% CONTROL
120
100
80
60
40
20
0
.01
.1
1
10
100
[VINCRISTINE] μM

FIG.7

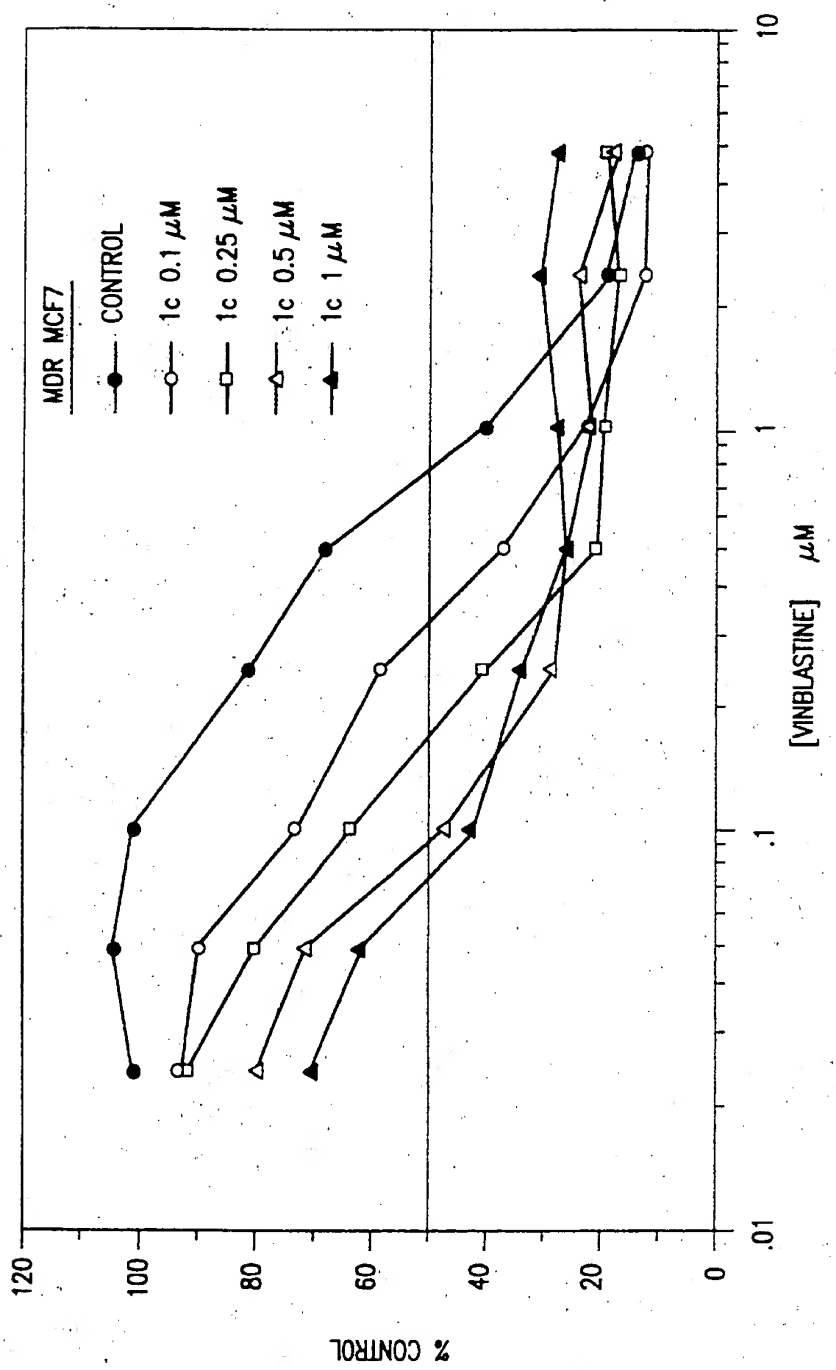

MDR MCF7
CONTROL
1c 0.1 μM
1c 0.25 μM
1c 0.5 μM
1c 1 μM
% CONTROL
120
100
80
60
40
20
0
.01
.1
1
10
[VINBLASTINE] μM

FIG.8

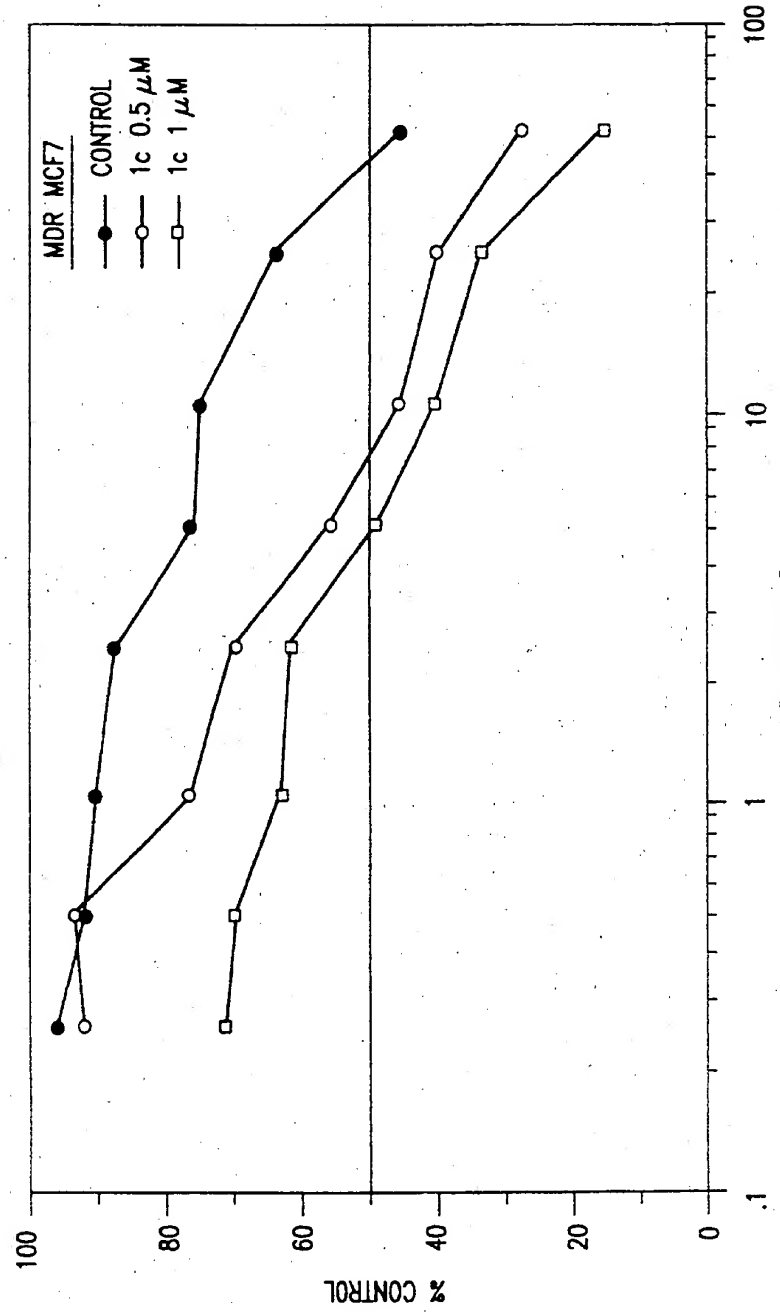

MDR MCF7
CONTROL
1c 0.5 μM
1c 1 μM
% CONTROL
100
80
60
40
20
0
.1
1
10
100
[ADRIAMYCIN] μM

FIG.9

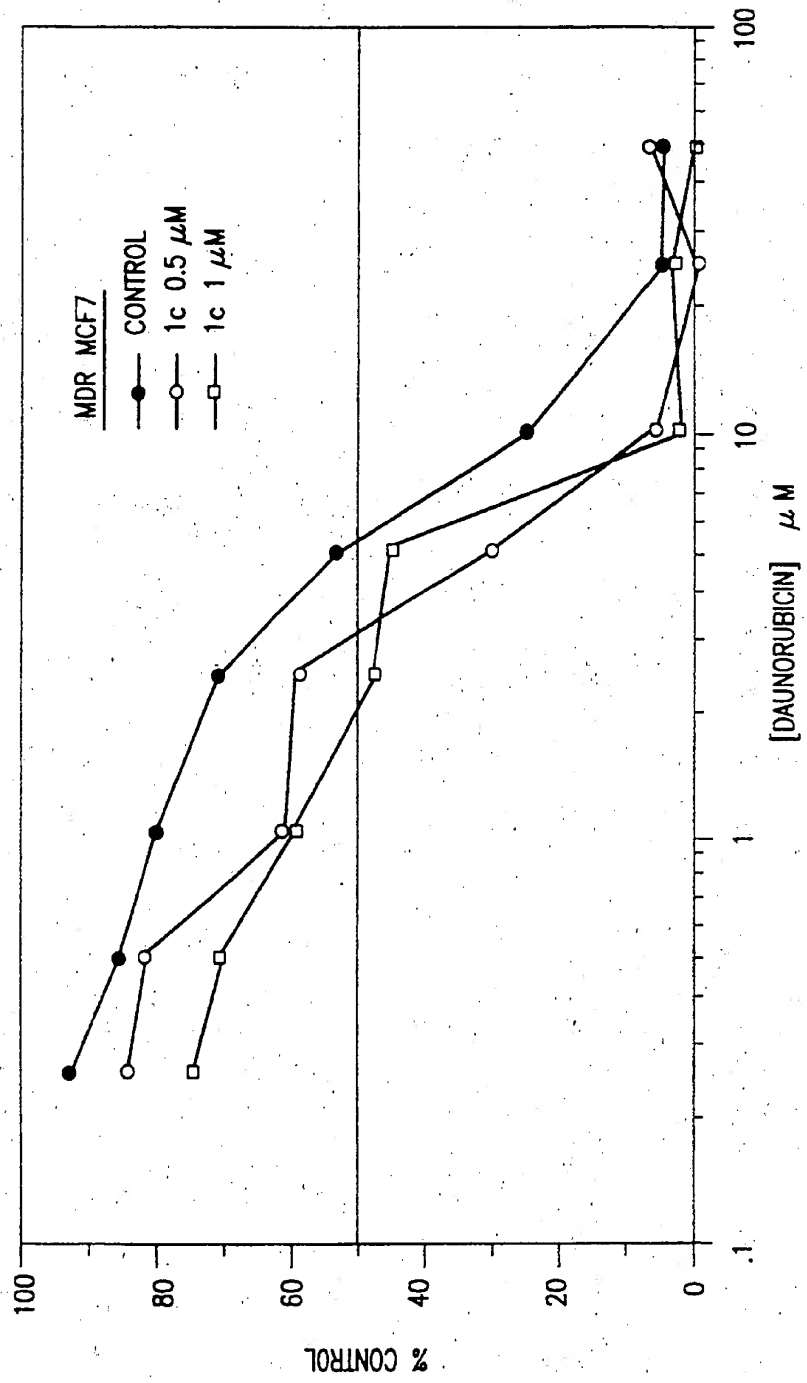

MDR MCF7
CONTROL
1c 0.5 μM
1c 1 μM
% CONTROL
100
80
60
40
20
0
.1
1
10
100
[DAUNORUBICIN] μ M

FIG.10

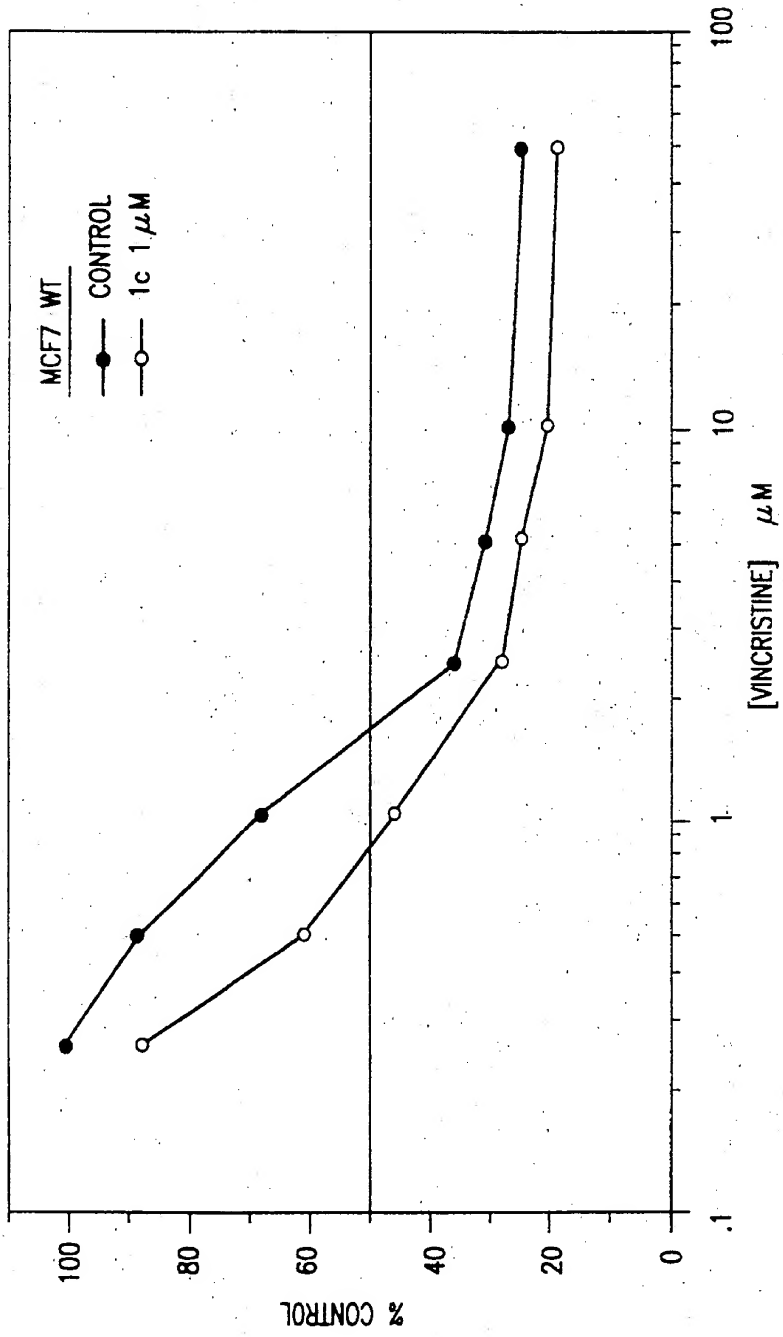

MCF7 WT
CONTROL
1c 1 μM
% CONTROL
100
80
60
40
20
0
.1
1
10
100
[VINCRISTINE] μM

FIG. 11

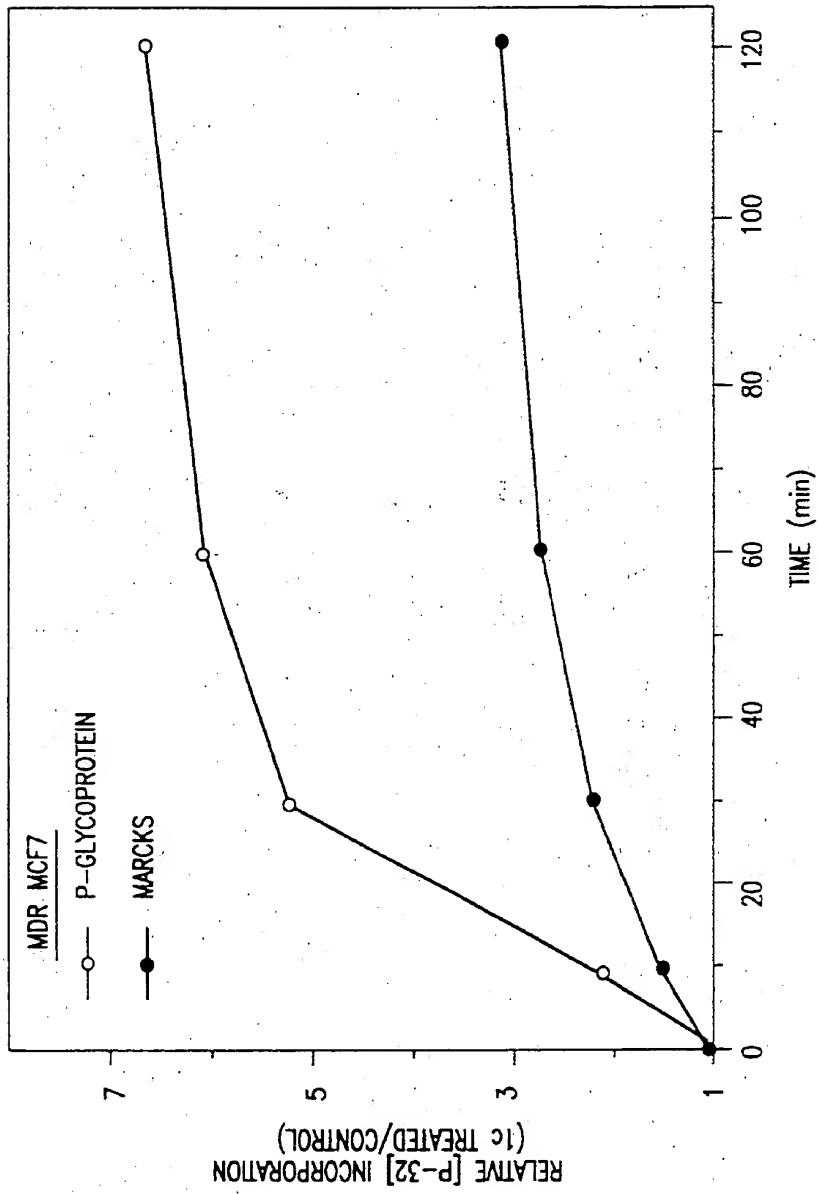

MDR MCF7
P-GLYCOPROTEIN
MARCKS
RELATIVE [P-32] INCORPORATION
(c TREATED/CONTROL)
TIME (min)
0
20
40
60
80
100
120
1
3
5
7

FIG.12

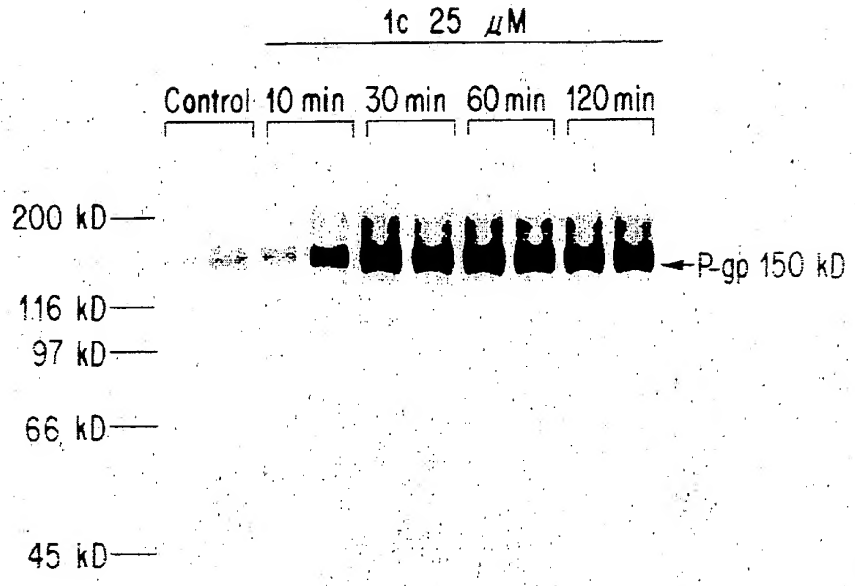

1c 25 μM
Control
10 min
30 min
60 min
120 min
200 kD
116 kD
97 kD
66 kD
45 kD
P-gp 150 kD

FIG. 13

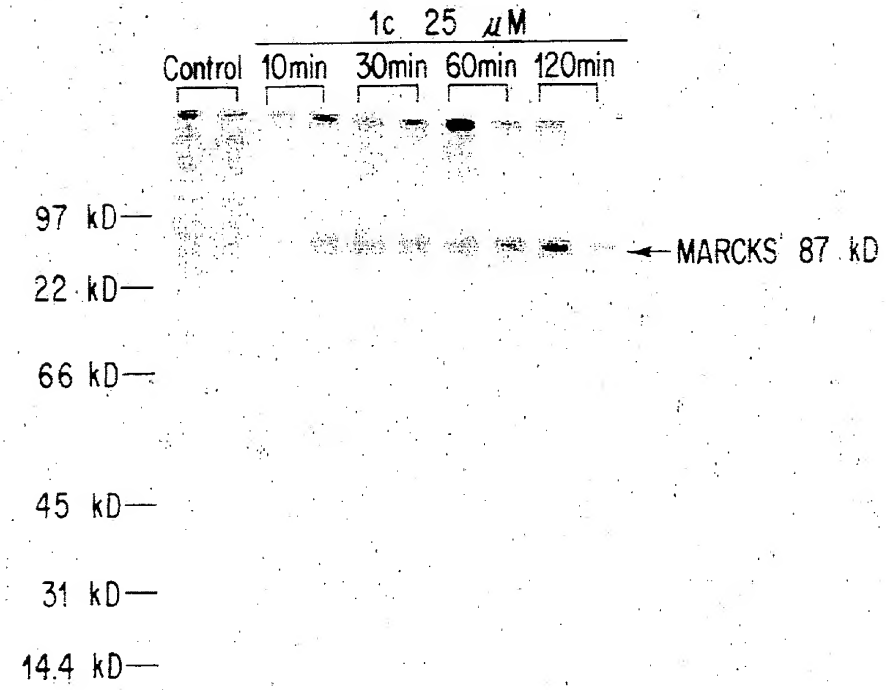

1c 25 μM
Control
10min
30min
60min
120min
97 kD
22 kD
66 kD
45 kD
31 kD
14.4 kD
MARCKS 87 kD

FIG. 14

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,436,243
DATED : July 25, 1995
INVENTOR(S) : Clifford W. Sachs, Robert L. Fine, Lawrence M. Ballas, F. Ivy Carroll, and Robert Bell

It is certified that error appears in the above-indentified patent and that said Letters Patent is hereby corrected as shown below:

ON THE TITLE PAGE

Item [75], delete the initial "R" in the name of the fourth inventor and replace with the initial "F".

Item [73], the assignee should read as follows:
--Duke University and Sphinx Pharmaceuticals Corporation, both of Durham, N.C.; Research Triangle Institute, Research Triangle Park, N.C.--.

Signed and Sealed this

Twenty-fourth Day of October, 1995

Attest:

BRUCE LEHMAN

Attesting Officer

Commissioner of Patents and Trademarks

AMINOANTHRAQUINONE DERIVATIVES TO COMBAT MULTIDRUG RESISTANCE

This invention was made with government support under Grant No. 5UO1-CA-46738 awarded by the National Cancer Institute. Research laboratories supported by the Durham Veterans Administration Medical Center were used in the performance of this research. The government has certain fights in this invention.

FIELD OF THE INVENTION

The present invention relates to the use of N-substituted alkyl amino anthraquinones and salts thereof as chemotherapeutic agents for neoplasms resistant to multiple drugs. The present invention more particularly relates to the use of such compounds as agents for enhancing the therapeutic effect of other antitumor agents.

BACKGROUND OF THE INVENTION

Complete cures of various tumors like leukemias, lymphomas and solid tumors by the use of chemotherapeutic agents are not frequently achieved because of heterogeneous sensitivity of tumor cells to each antitumor agent. Cancer chemotherapy also fails because of intrinsic resistance of the tumor to various drug therapies. In other cases, a tumor may become resistant to the antitumor agents used in a previous treatment. The therapeutic effects of these agents are then lost. An even graver problem is that recurrent and relapsed cancers are resistant not only to the anticancer drugs used in previous treatments, but also manifest resistance to other antitumor agents, unrelated to the drug used previously either by chemical structure or by mechanism of action. These phenomena are collectively referred to as multidrug resistance.

The emergence of drug resistance represents a major obstacle to the successful treatment of cancers which can be treated by chemotherapy. To circumvent the problem of clinical drug resistance, chemotherapy may utilize combinations or successive treatments with functionally and structurally diverse antineoplastic agents to minimize the development of drug resistance and maximize the response to therapy. Despite this approach, most cancer patients relapse or never respond because of the development of drug resistance and further responses to therapy are limited (Chabner et. al., *Cancer* 54:2599-2608 (1984)).

The origins of tumor cell drug resistance in cancers which initially respond to chemotherapy are not fully understood. According to one theory, the somatic mutation theory, tumor cells are characterized by genetic instability (Goldie et al., *Cancer Res.* 44:3643-3653 (1984)), with observed frequencies of mutation to drug resistance of one in 10^5 to 10^7 (Bellamy et at., *Cancer Invest.* 8:547-562 (1990)). Since a detectable tumor mass of 1 cm^2 (approximately 1 g) contains at least 10^9 cells, it is nearly certain that some of the cells are resistant at the time of diagnosis prior to chemotherapy (Id.).

Cancer cells may become refractory to chemotherapy by several mechanisms. One type of drug resistance, called multidrug resistance, arises in cancer cells exposed to anticancer drugs derived from natural products (i.e., antineoplastic agents isolated from plants, fungi or bacteria). It can develop in cancer cells exposed to a single natural antineoplastic drug. In multidrug resistant (MDR) cells, cross resistance is observed to natural product antineoplastic agents (Vinca alkaloids, anthracyclines, epipodophyllotoxins, colchicine, actinomycin D and antibiotics) (See I. Pastan and M. Gottesman, *New England J. Med.* 1388, 1389 Table 1 (May 28, 1987)), but not alkylating anticancer drugs, bleomycin, or antimetabolites. The MDR phenotype is characterized by: (1) decreased intracellular accumulation of natural product anticancer drugs, secondary to their enhanced efflux; (2) cross resistance to other structurally and functionally unrelated natural product antineoplastic drugs; and (3) overexpression of a high molecular weight (150-170 kilodalton) transmembrane protein, termed the P-glycoprotein (the multiple drug transporter) which acts as drug transport pump. P-glycoprotein is an ATPase which functions by pumping structurally diverse antitumor drugs from cells. (See R. Fine and B. Chabner, *Multidrug Resistance, in Cancer Chemotherapy*, 117-128 (H. Pinedo and B. Chabner eds. 1986)(reviewed in Fine et al., Multidrug Resistance, Cancer Chemotherapy and Biological Response Modifiers, Eds: Pinedo, HM, Longo, DL and Chabner, BA. Elsvier Scientific Publications, NY, NY (1988)); Moscow et al., *J. National Cancer Institute,* vol. 80, 14-20 (1988); and Ford et al., *Pharmacol. Rev.* 42:155-199 (1990)).

A number of studies have implicated the P-glycoprotein in the MDR phenotype. The presence of the P-glycoprotein generally correlates with resistance in MDR cell lines (Kartner et al., *Science* 221:1285-1288 (1983)). The degree of resistance of certain tumor cells has been documented to correlate with both elevated expression of the drug transporter and reduced accumulation of antitumor drugs. (See A. Fojo et al., *Cancer Res.* 45:3002-3007 (1985).) Tumor cells expressing elevated levels of the multiple drug transporter accumulate far less antitumor agents intracellularly than tumor cells having low levels of the P-glycoprotein. Further, drug sensitive cells stably transfected with the mdr1 gene overexpress the P-glycoprotein and exhibit the MDR phenotype (Schurr et al., Cancer Res 49:2729-2734 (1989); Hammond et al., *Cancer Res.* 49:3867-3871 (1989); Sugimoto et al., *Cancer Res.* 47:2720-2726 (1987)). The role of P-glycoprotein as an energy dependent efflux pump is supported by the findings that depletion of cellular ATP in MDR cells eliminates the reduced drug accumulation defect of MDR cells (Dano, *Biochimica et Biophysica Acta* 323:466-483 (1973)), that the purified P-glycoprotein has ATPase activity (Hamada et al., *J. Biol. Chem.* 263:1454-1458 (1988)), and that expression of human DNA coding for P-glycoprotein confers high activity drug stimulatable ATPase activity (Sarkadi, B. et al., *J. Biol. Chem.* 267:4854-4858, 1992). Using antibodies to the P-glycoprotein, membrane vesicles prepared from MDR cell lines have been shown to contain this protein which is absent from membrane vesicles of parental drug sensitive cell lines by Western blot methods. The P-glycoprotein found in membrane vesicles of MDR cells shows specific binding of radiolabeled anticancer drugs and photoactive drug analogs. Membrane vesicles prepared from drug sensitive cell lines do not show specific binding of these compounds (Cornwell et al., *J. Biol. Chem.* 261:7921-7928 (1986); Cornwell et al., *Proc. Natl. Acad. Sci., USA* 83:3847-3850 (1986); Naito et al., *J. Biol. Chem.* 263:1187-11891 (1989)). Nucleotide sequence analysis of the mdr1 gene indicates that it codes for a 1280 amino acid protein with 12 transmembrane regions and 2 nucleotide binding sites. The deduced

amino acid sequence of the P-glycoprotein shows extensive homology with the bacterial membrane transport protein for hemolysin B (Gerlach et al., *Nature* 324:485–489 (1986); Gros et al., *Cell* 47:371–380 (1986); Chen et al., *Cell* 47:381–389 (1986)).

A role for the P-glycoprotein in clinical drug resistance is suggested by several studies which have utilized specific monoclonal antibodies to the P-glycoprotein or cDNA probes to measure mdr1 RNA levels in tumors. High levels of the P-glycoprotein have been detected in drug-refractory hematologic malignancies, ovarian cancers, neuroblastomas, and sarcomas. These cancers usually respond initially to chemotherapy, but become refractory to further treatment and are considered to have acquired resistance. Other tumors documented to initially be drug-sensitive but to then become drug resistant include pheochromocytoma, acute lymphocytic leukemia in adults, acute nonlymphocytic leukemia in adults, nodular poorly differentiated lymphoma and breast cancer. High levels of the P-glycoprotein have also been found in untreated colon, renal, adrenal, and hepatic carcinomas which do not respond well to chemotherapy (reviewed in Bellamy et al., *Cancer Invest.* 8:547–562 (1990)). Interestingly, P-glycoprotein RNA expression is high in normal kidney, adrenal, hepatic and colonic tissues (Fojo et al., *Proc. Natl. Acad. Sci., USA,* 84:265–269 (1987)). These tissues have major roles in detoxification and secretion of toxins, suggesting a possible protective physiological role for the P-glycoprotein in normal tissues. Adult tumors derived from these tissues usually do not respond to chemotherapy. These types of tumors are considered to have intrinsic resistance. Other tumors documented to express high levels of the multidrug transporter include pancreatic, carcinoid, and chronic myelogenous leukemia in blast crisis. Increased levels of mdr1 expression have been found in untreated tumors derived from the colon, liver, kidney, adrenal gland, and pancreas which are considered to be intrinsically resistant (Goldstein et al., *J. Natl. Cancer Inst.* 81:116–124 (1989)).

It is likely that several mechanisms other than reduced drug accumulation may play a role in drug resistance. These include differences in DNA repair capacities, increased detoxification of anticancer drugs, alterations of the drug targets, and alterations of subcellular distributions of anticancer drugs which decrease drug concentrations at their targets (Fine, "Multidrug Resistance, Cancer Chemotherapy and Biological Response Modifiers", Eds: Pinedo, HM, Longo, DL and Chabner, BA. Elsvier Scientific Publications, NY, NY (1988); Moscow et al., *J. National Cancer Institute,* vol. 80, 14–20 (1988); Ford et al., *Pharmacol. Rev.* 42:155–199 (1990); Endicott et al., *Annual Rev. Biochem.* 58:137–171 (1989)). However, the P-glycoprotein is considered to be the major determinant of the MDR phenotype.

There is substantial evidence to suggest that P-glycoprotein function can be modulated by phosphorylation of the P-glycoprotein by cellular kinases, notably protein kinase C (PKC). Many MDR phenotype inhibitors are also PKC inhibitors. A number of laboratories have reported increased PKC activity in MDR cell lines and an association between PKC stimulation and the MDR phenotype (Palayoor et al. Biochem & Biophys. Res. Comm. 148:718–721 (1987); Fine et al., *Proc. Natl. Acad. Sci., USA* 85:582–587 (1988); Posada et al., *Cancer Res.* 49:6634–6640 (1989); Ferguson et al., *Cancer Res.* 47:433–441 (1987); O'Connor et al., Leuk. Res. 9:885–895 (1985); O'Brian et al., *FEBS Lett.* 246: 78–82 (1989); and Posada et al., Cancer Commun. 1:285–292 (1989)). In the MCF7 cell line, activation of PKC by phorbol ester has been shown to reduce intracellular accumulation of vincristine and doxorubicin, to transiently induce the MDR phenotype in sensitive, wild type (MCF7 wt) cells and to increase the MDR phenotype in the MDR MCF7 (MCF7 Adr 10) cells (Fine et al., *Proc. Natl. Acad. Sci., USA* 85:582–587 (1988)). The P-glycoprotein has been shown to be phosphorylated by a number of cellular kinases, including PKC (Yu et al., *Cancer Commun.* 3:181–189 (1991); and Chambers et al., *J. Biol. Chem.* 265:7679–7686 (1990)). Interestingly, phorbol esters, and verapamil, a calcium channel blocker, inducers and an inhibitor of the MDR phenotype, respectively, increased phosphorylation of the P-glycoprotein on different serine residues of the protein (Hamada et at., *Cancer Res.* 47:2860–2865 (1987); and Chambers et al., *J. Biol. Chem.* 265:7679–7686, 1990). Also interestingly, a cell line that was transfected with copies of the mdr1 gene did not express an MDR phenotype equivalent to adriamycin selected cells, yet expressed equivalent amounts of P-glycoprotein. When these cells were transfected with the α isoenzyme of PKC, their level of the MDR phenotype increased substantially, and was associated with P-glycoprotein phosphorylation and decreased drug accumulation (Yu et at., *Cancer Commun.* 3:181–189 (1991)). Reversal of the MDR phenotype probably involves multiple mechanisms, including inhibition of anticancer drug binding to P-glycoprotein, inhibition of P-glycoprotein efflux, and changes in P-glycoprotein phosphorylation.

There are two major strategies to overcome the problem of the MDR phenotype. One approach is to create new antineoplastic drugs or develop analogues of antineoplastic drugs currently used which are cytotoxic to MDR cancer cells. Two examples of anticancer drugs to which some MDR cells show less cross-resistance are mitoxantrone (Novantrone; see U.S. Pat. No. 4,197,249), a new antineoplastic agent, and morpholino anthracycline analogues of adriamycin and daunomycin. (Coley et at., *Cancer Chemother. Pharmacol.* 24:284–290 (1989)). The second approach to overcome the problem of the MDR phenotype is to identify resistance modifiers which reduce the degree of resistance in MDR cell lines in vitro. Such modifiers would be agents that inhibit the active efflux of antitumor agents by the drug transporter and/or agents that potentiate the efficacy of chemotherapeutic agents. The pharmacology and biochemistry of these types of resistance modifiers are extensively reviewed in Ford et at., *Pharmacol. Rev.* 42:155–199 (1990) and briefly summarized below for the most promising major resistance modifiers. These compounds are being or have been tested in clinical trials. They include calcium channel blockers, calmodulin antagonists, cyclosporins, and hormonal analogs.

Verapamil, a calcium channel blocker, was the first described resistance modifier and is probably the most potent in vitro resistance modifier previously known. Numerous investigators have described increases in accumulation and decreases in resistance to natural product chemotherapeutic drugs in a number of different MDR cells treated with verapamil and other calcium channel blockers. The mechanism by which verapamil, other calcium channel blockers, and calmodulin antagonists are thought to increase drug accumulation is by competing with anticancer drugs for binding to the P-glycoprotein, thereby inhibiting efflux

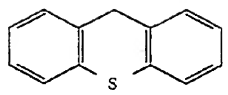
of drug (Cornwell et al., *J. Biol. Chem.* 261:7921-7928 (1986); Cornwell et at., *J. Biol. Chem.* 262:2166-2170 (1986); Safa et at., *J. Biol. Chem.* 7884-7888 (1987); and Akiyama et at., *Mol. Pharmacol.* 33:144-147 (1988)).

Verapamil has been tested in Phase I and Phase II clinical trials (Benson et al., *Cancer Treat Rep.* 69:795-799 (1985); Preasant et al., *Am. J. Clin. Oncol.* 9:355-357 (1986); and Ozols et at., *J. Clin. Oncol.* 5:641-647 (1987)). However, the major problem associated with using verapamil to reverse drug resistance in patients is that it has dose-limiting cardiac toxicity due to blocking of the atrioventricular node. This toxicity prevents its use at concentrations required to reverse drug resistance in vitro. Thus, the lack of response observed in those studies may stem from the inability to achieve high enough concentrations to modulate clinical drug resistance. In a recent study, 3 of 6 patients with clinically resistant, P-glycoprotein positive myeloma responded to a regimen consisting of continuous infusion vincristine, adriamycin (i.e., doxorubicin) plus oral dexamethasone (VAD regimen) when verapamil was included in the regimen by continuous I.V. infusion. These patients had progressive disease while on the VAD regimen prior to the addition of verapamil (Dalton et at., *J. Clin. Oncol.* 7:415-424 (1989)).

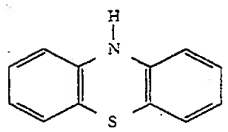
Most studies of verapamil's effects on multidrug resistance have utilized racemic mixtures of the drug. The L form of verapamil is 10 times more active as a calcium antagonist than the D form of this compound, although both forms of verapamil increase drug accumulation to a similar extent (Gruber et al., Int. J. Cancer 41:224-229 (1988); and Mickish et at., Cancer Res. 50:3670-3674 (1990)). The use of the D form of this compound is an approach which may circumvent the obstacle of cardiovascular toxicity of calcium channel blockers, such as verapamil. However, more recent studies suggest that even D-verapamil is too toxic to use at high concentrations in humans.

A number of calmodulin antagonists have been found to be good resistance modifiers in vitro. Trifluoperazine, a phenothiazine antipsychotic drug, has been noted to increase drug accumulation and decrease resistance in MDR cell lines (Tsuruo et al., *Cancer Res.* 43:2905-2910 (1983); and Ford et al., *Mol. Pharmacol* 35:105-115 (1989)). Although the increases in drug accumulation were comparable to verapamil in these studies, verapamil was more effective in reversal of resistance to anticancer drugs in other studies (Tsuruo et at., *Cancer Res.* 43:2905-2910 (1983); and Ford et al., *Mol. Pharmacol.* 35:105-115 (1989)). In a clinical trial that combined oral trifluoperazine with constant infusion of adriamycin, 36 patients with tumors clinically resistant to adriamycin were treated. One complete response and six partial responses were noted. Neurotoxicity of trifluoperazine to the extrapyramidal tracts was dose-limiting. The plasma concentrations of trifluoperazine were approximately 10-fold less than the concentrations found to be optimal for modulation of chemoresistance in vitro (Miller et at., J. Clin. Oncol. 6:880-888 (1990)).

The thioxanthene class of antipsychotic drugs are similar in structure to the phenothiazines. The similarity is apparent when comparing the structures of thioxanthene and phenothiazine:



Thioxanthene



Phenothiazine

A number of these compounds have recently been evaluated for the ability to reverse the MDR phenotype in MDR cell lines (Miller et at., *J. Clin. Oncol.* 6: 880-888, 1990.). Trans-flupenthixol, a thioxanthene, increased doxorubicin accumulation and reversed the MDR phenotype more effectively in a number of MDR cell lines than verapamil (Ford et at., *Cancer Res.* 50:1748-1756 (1990)). The trans isomer of flupenthixol was a much less potent antipsychotic in clinical trials than the cis isomer of this compound (Ford et at., Cancer Res 50:1748-1756 (1990)). It may be a more promising resistance modifier than trifluoperazine if it has less neurotoxicity.

The cyclosporins are immunosuppressive agents which can also potentiate toxicity of anticancer agents at clinically achievable concentrations. In some cell lines, it appears to enhance toxicity and increase drug accumulation in sensitive and resistant cells (Chambers et al., *Cancer Res.* 49:6275-6279 (1989)). Cyclosporin A (CsA) competitively inhibits vincristine and vinblastine binding to the P-glycoprotein (Tamai et at., *J. Biol. Chem.* 265:16509-16513 (1990)). Both Cylcosporin A and its nonimmunosuppressive analog, O-acetyl C_9-cyclosporin A (SDZ 33-243) inhibit [^{3}H] azidopine photoaffinity labeling of P-glycoprotein in intact MDR cells and in MDR membrane vesicles (Tamai et at.. *J. Biol. Chem.* 266:16796-16800 (1991)). The use of cyclosporin A to modulate drug resistance may be hampered by irreversible nephrotoxicity and immunosuppression in patients already compromised by myelosuppressive chemotherapy, whereas the use of nonimmunosuppresive cyclosporin analogs may be less toxic.

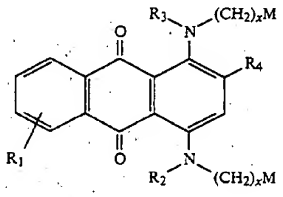
Hormonal analogs such as the antiestrogens tamoxifen and toremifene are employed in the chemotherapy of breast cancers. These compounds can also modulate resistance of estrogen receptor-negative MDR cell lines via estrogen receptor independent mechanisms (Ramu et at., *Cancer Res.* 44:4392-4395 (1984); and Bermin et al., *Blood* 77:818-825 (1991)). Tamoxifen and tamoxifen metabolites have been found to increase drug accumulation and decrease vinblastine resistance in intrinsically resistant renal cell carcinoma cell lines (Fine et al., Proceedings of the AACR Annual Meeting Abstract #2125 (1990)). The concentrations which elicited these responses were comparable to plasma concentrations achieved in patients participating in Phase I clinical trials to tamoxifen conducted at Duke University, which concentrations were associated with minimal neurologic toxicity that was reversible with cessation of tamoxifen and partial responses to the vinblastine-

tamoxifen regimen (Trump, E. L. *J. Natl. Cancer Inst.* 84: 1811–1816, 1992).

In summary, a number of studies have demonstrated that multidrug resistance mediated by the function of the P-glycoprotein occurs in both cultured cell lines and human cancers. The identification of several pharmacologic agents which can antagonize the MDR phenotype in the laboratory has not, to date, identified resistance modifiers with good clinical efficacy, primarily due to doselimiting toxicity of the resistance modifiers. Thus there is a need in the art for means to overcome multidrug resistance expressed by tumors and for means to potentiate the effects of chemotherapeutic agents, in general.

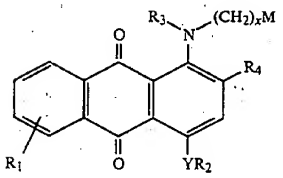
SUMMARY OF THE INVENTION

An object of the present invention is to provide pharmaceutical formulations for enhancing the therapeutic effect of antineoplastic agents by administering to a subject harboring a tumor a compound of Formula (I) or Formula (II) or a pharmaceutically acceptable salt thereof or combinations thereof (hereafter referred to as the "potentiating agent"):



Formula I

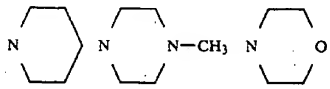
and

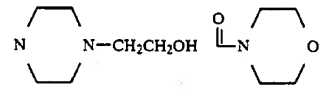


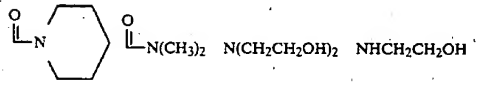
Formula II

wherein:

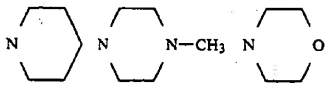
R_1 is H or an alkyl chain with 1–7 carbon atoms;
R_2 is H or an alkyl chain with 1–7 carbon atoms;
R_3 is H or an alkyl chain with 1–7 carbon atoms;
R_4 is H or an alkyl chain with 1–7 carbon atoms;
x is an integer ranging from 3 to 12;
Y is O or NH; and
M is selected from the following groups:

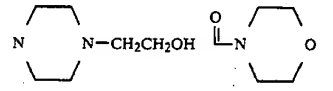


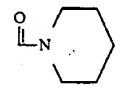




Preferably the potentiating agents are those wherein M is selected from the following groups:







More preferably, the potentiating agents are compounds according to Formula I or a pharmaceutically acceptable salt thereof or combinations thereof wherein R_1, R_2, R_3, and R_4 are H.

The pharmaceutical formulation containing a potentiating agent may optionally contain an antineoplastic agent in a pharmaceutically acceptable carder.

Another aspect of the present invention is a method of increasing the sensitivity of tumor cells to an antineoplastic agent to which the tumor cells are resistant, by administering to the resistant tumor cells a potentiating agent concurrently or sequentially with an antineoplastic agent. Resistance to the antineoplastic agent may (a) be an intrinsic property of the tumor cells or Co) develop in response to prior treatment with said antineoplastic agent or another antineoplastic agent capable of selecting for the MDR phenotype.

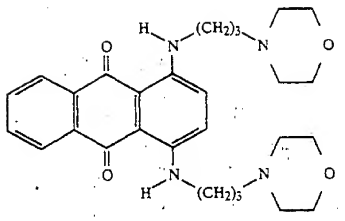
According to another aspect of the invention a method is provided for selectively inhibiting the growth of tumor cells in a subject in need of such treatment. An antineoplastic agent and a potentiating agent are concurrently or sequentially administered to the subject. The potentiating agent is administered in an amount effective to (a) reduce the amount of the antineoplastic agent required to achieve a growth inhibiting effect on the tumor cells relative to the amount required to achieve the same effect by antineoplastic agent without administration of the potentiating agent; or (b) inhibit the development of the multidrug resistance phenotype in tumor cells.

Yet another aspect of the invention is the use of compounds of Formula I for the sensitization of tumor cells to Vinca alkaloids.

Another aspect of the present invention is the use of a potentiating agent as disclosed herein for the manufacture of a medicament for the inhibition of the multidrug resistance in tumors.

As part of the present invention, alkyl aminoanthraquinones have been indentified as inhibitors of the MDR phenotype in a human breast cancer cell line, MCF7 Adr 10. One such potent compound is 1,4-bis-[3'-(N-morphilino)propyl amino]anthraquinone:

Compound 1c



In contrast to most of the previously described multidrug resistance inhibitors which have activity at micromolar concentrations, Compound **1***c* has been found to inhibit Vinca alkaloid drug resistance at nanomolar concentrations. Compound **1***c* also inhibits resistance to the anthracycline antineoplastic drugs, adriamycin and daunorubicin, in MDR cells and enhances toxicity of vincristine in the parental, drug sensitive, breast cancer cell line, MCF7 WT. Compound **1***c* has been shown to modulate the drug accumulation defect of a MDR human breast cancer cell line and intrinsically resistant, human, renal cell carcinoma cell lines. In all of these systems, its activity has been found to be comparable or superior to that of verapamil, a previously described, resistance modifier. Compound **1***c* has a large therapeutic index for bone marrow cytotoxicity versus cancer cell cytotoxicity. Preliminary animal studies show that compound **1***c* is not toxic at 400 mg/kg in mice.

IN THE DRAWINGS

FIG. **1** is a graph of the time course of accumulation of Compounds **1***b* and **1***c* in MCF7 Adr 10 human breast cancer cells.

FIG. **2** is a bar graph Of the activities of vehicle controls, Compound **1***c* and verapamil, respectively, in MCF7 Adr 10 vinblastine accumulation assays.

FIG. **3** is a bar graph of the activities of vehicle controls, Compounds **1***b* and **1***d*, and verapamil, respectively, in MCF7 Adr 10 vinblastine accumulation assays.

FIG. **4** is a bar graph of the activity of a vehicle control, Compounds **1***c* and verapamil, respectively, in A704 human renal cell line in vinblastine accumulation assays.

FIG. **5** is a bar graph of the activity of a vehicle control, Compound **1***c* and verapamil, respectively, in SKRC7 human renal cell line in vinblastine accumulation assays.

FIG. **6** is a bar graph of the activity of a vehicle control, Compound **1***c* and verapamil, respectively, in UOK-39 human renal cell line in vinblastine accumulation assays.

FIG. **7** is a graph of inhibition concentration (IC) curves for vincristine in MCF7 Adr 10 cell line which shows the potentiating effect on vincristine toxicity as a function of Compound **1***c* concentration.

FIG. **8** is a graph of IC curves for vinblastine in MCF7 Adr 10 cell line which shows the potentiating effect on vincristine toxicity as a function of Compound **1***c* concentration.

FIG. **9** is a graph of IC curves for adriamycin (doxorubicin) in MCF7 Adr 10 cell line which shows the potentiating effect on doxorubicin toxicity as a function of Compound **1***c* concentration.

FIG. **10** is a graph of IC curves for daunorubicin in MCF7 Adr 10 cell line which shows the potentiating effect on daunorubicin toxicity as a function of Compound **1***c* concentration.

FIG. **11** is a graph of IC curves for vincristine in MCF7 WT cell line which shows the potentiating effect on vincristine toxicity as a function of Compound **1***c* concentration.

FIG. **12** is a graph of the time course of Compound **1***c* stimulated phosphorylation of P-glycoprotein and MARCKS, respectively, in MDR MCF7 cells

FIG. **13** is an audioradiograph of the time course of Compound **1***c* stimulated phosphorylation of P-glycoprotein in MCF7 Adr 10 cell line.

FIG. **14** is an autoradiograph of the time course of Compound **1***c* stimulated phosphorylation of MARCKS in MCF7 Adr 10 cell line.

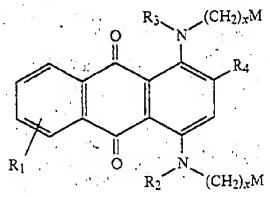
DETAILED DESCRIPTION OF THE INVENTION

It has now been found that when the agents disclosed herein are used together with an antitumor agent, they enhance the therapeutic effect of the antitumor agent. It is believed that the N-substituted alkyl amino anthraquinones utilized in the present invention are better suited for clinical application than previously described resistance modifiers.

While the inventors do not wish to be bound by any theory of operation for the present invention, it is noted that the agents disclosed herein are not known to be calcium-channel blockers or calmodulin antagonists. They have not shown in vitro inhibitory activity against a number of cellular kinases. They have, however, been found to elevate the intracellular concentration of antineoplastic drugs in tumor cells overexpressing the multiple drug transporter. Sensitization of drug resistant tumors and elevation of intracellular antitumor drug concentrations probably occur by a mechanism different from calcium antagonism.

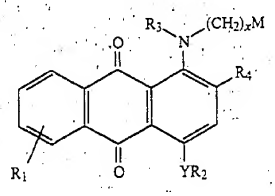
Potentiating agents disclosed herein include:

Formula I



and

Formula II



wherein:

R_1 is H or an alkyl chain with 1–7 carbon atoms;

R_2 is H or an alkyl chain with 1–7 carbon atoms;

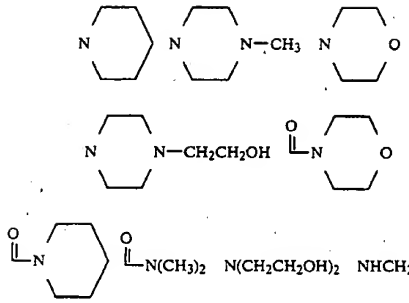
R_3 is H or an alkyl chain with 1–7 carbon atoms;

R_4 is H or an alkyl chain with 1–7 carbon atoms;

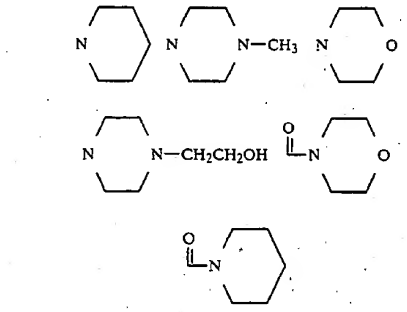
x is an integer ranging from 3 to 12;

Y is O or NH; and

M is selected from the following groups:



Preferably the potentiating agents are those wherein M is selected from the following groups:



More preferably, the potentiating agents are compounds according to Formula I or a pharmaceutically acceptable salt thereof; wherein R_1, R_2, R_3, and $R_4$are H.

Salts of the foregoing compounds useful in the present invention are acid addition salts, especially pharmaceutically useful nontoxic acid addition salts, such as those with inorganic acids, for example, hydrochloric, hydrobromic, nitric, sulfuric, or phosphoric acid, or with organic acids, such as organic carboxylic acids, for example, acetic, propionic, glycolic, malonic, succinic, maleic, hydroxymaleic, methylmaleic, fumaric, malic, tartaric, citric, benzoic, cinnamic, manrelic, salicylic, 4-aminosalicylic, 2-phenoxybenzoic, 2-acetoxybenzoic, embonic, nicotinic or isonicotinic, or organic sulfonic acids, for example, methanesulfonic, ethanesulfonic, 2-hydroxy-ethanesulfonic (isethionic), ethane-1,2-disulfonic, benzenesulfonic, p-toluenesulfonic, naphthalene-2-sulfonic, or cyclohexane sulfamic acid, as well as ascorbic acid.

A preferred category of drug resistant tumor cells to be treated by the method of the present invention are MDR cells, which contain P-glycoprotein, the multidrug transporter protein described in M. Gottesman and J. Pastan, supra. Thus, tumor cells treated by the present invention are preferably those characterized by (a) the expression of the multidrug transporter protein, or (b) the ability to express the multidrug transporter protein upon selection by an antineoplastic agent.

Exemplary of tumor cells which express the multidrug transporter (intrinsically resistant cells) are adenocarcinoma cells, pancreatic tumor cells, pheochromocytoma cells, carcinoid tumor cells, chronic myelogenous leukemia cells in blast crisis, renal cell, hepatocellular tumor cells, adrenal cancer cells, and colon cancer cells. Other tumor cell types may also be treated by the potentiating agents of the present invention and sensitized thereby to one or more antineoplastic agents.

Exemplary of tumor cells having the ability to express the multidrug transporter protein upon selection by an antineoplastic agent are neuroblastoma cells, adult acute lymphocytic leukemia cells, adult acute nonlymphocytic leukemia cells, nodular poorly differentiated lymphoma cells, breast cancer cells and ovarian cancer cells. A preferred group of tumor cells for treatment in the present invention are the adenocarcinomas, including adenocarcinomas of adrenal, kidney, liver, small intestine and colon tissue.

Preferred antineoplastic agents for use in the present invention are those to which multidrug transporter-mediated MDR cells develop resistance. Exemplary of such antineoplastic agents are Vinca alkaloids, epipodophyllotoxins, antibiotics, anthracycline antibiotics, actinomycin D, puromycin, gramicidin D, taxol, taxotere, colchicine, topoisomerase I and II inhibitors, cytochalasin B, emetine, maytansine, and amsacrine (or "mAMSA"). Preferred antineoplastic agents are Vinca alkaloids, epipodophyllotoxins, anthracycline antibiotics, actinomycin D, taxol, taxotere, and topoisomerase I inhibitors (camptothecin and topotecan).

The Vinca alkaloid class is described in *Goodman and Gilman's The Pharmacological Basis of Therapeutics,* 1277–1280 (7th ed. 1985) (hereafter "Goodman and Gilman"). Exemplary of Vinca alkaloids are vincristine, vinblastine, and vindesine.

The epipodophyllotoxin class is described in Goodman and Gilman, supra. at 1280–1281. Exemplary of epipodophyllotoxins are etoposide, etoposide orthoquinone, etoposide phosphate, and teniposide.

The anthracycline antibiotic class is described in Goodman and Gilman, supra. at 1283–1285. Exemplary of anthracycline antibiotics are daunorubicin, doxorubicin, mitoxantrone, bisantrene, epirubicin, and idarubicin. Actinomycin D, also called Dactinomycin, is described in Goodman and Gilman, supra. at 1281–1283.

The phrase "concurrently administering," as used herein, means that the antineoplastic agent and the potentiating agent are administered either (a) simultaneously (optionally by formulating the two together in a common carrier), or (b) at different times during the course of a common treatment schedule. In the latter case, the two compounds are administered at times sufficiently close for the potentiating agent to enhance the selective growth-inhibiting action of the antineoplastic agent on the tumor cells. This may be within one month, one week, one day or one hour.

Subjects to be treated by the method of the present invention include both human and animal (e.g., dog, cat, cow, horse) subjects, and are preferably mammalian subjects.

The potentiating agent is administered in an amount effective to enhance the efficacy of the antineoplastic agent. The potentiating agent is preferably administered in a total amount per day of not more than about 1 g/kg body weight, more preferably not more than about 400 mg/kg, most preferably not more than about 50 mg/kg, and most preferably not more than 5 mg/kg. With respect to minimum dose, the potentiating agent is preferably administered in a total amount per day of at least about 0.01 mg/kg, more preferably at least about 0.1 mg/kg, and most preferably at least about 1 mg/kg. The

potentiating agent may be administered once or several times a day.

As noted above, the compounds of Formula (I) and (II) may be administered per se or in the form of a pharmaceutically acceptable salt. When used in medicine, the salts of the compound of Formula (I) and (II) should be both pharmacologically and pharmaceutically acceptable, but non-pharmaceutically acceptable salts may conveniently be used to prepare the free active compound or pharmaceutically acceptable salts thereof and are not excluded from the scope of this invention. Such pharmacologically and pharmaceutically acceptable salts include, but are not limited to, those prepared from the following acids, hydrochloric, hydrobromic, sulfuric, nitric, phosphoric, maleic, acetic, salicylic, p-toluenesulfonic, tartaric, citric, isethionic, methanesulphonic, formic, malonic, succinic, naphthalene-2-sulfonic and benzenesulphonic. Also, pharmaceutically acceptable salts can be prepared as alkaline metal or alkaline earth salts, such as sodium, potassium or calcium salts of the carboxylic acid group. Thus, the present invention also provides pharmaceutical formulations, both for veterinary and for human medical use, which comprise the potentiating agent together with one or more pharmaceutically acceptable carriers thereof and optionally any other therapeutic ingredients. The carrier(s) must be pharmaceutically acceptable in the sense of being compatible with the other ingredients of the formulation and not unduly deleterious to the recipient thereof.

Pharmaceutical formulations of the present invention may optionally include an antineoplastic agent, preferably an agent as described above. Such a formulation is useful for concurrently administering an antineoplastic agent and the potentiating agent in a method as described above.

The formulations include those suitable for oral, rectal, topical, nasal, ophthalmic, transdermal, or parenteral (including subcutaneous, intramuscular and intravenous) administration. Formulations suitable for oral and parenteral administration are preferred.

The formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. All methods include the step of bringing the active compound into association with a carrier which constitutes one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing the active compound into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product into desired formulations.

Formulations of the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets, tablets or lozenges, each containing a predetermined amount of the potentiating agent as a powder or granules. Formulations for oral administration also may be a suspension in an aqueous liquid or non-aqueous liquid such as a syrup, an elixir, an emulsion or a draught.

A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine, with the active compound being in a flee-flowing form such as a powder or granules which is optionally mixed with a binder, disintegrant, lubricant, inert diluent, surface active agent or dispersing agent. Molded tablets comprised of a mixture of the powdered active compound with a suitable carrier may be made by molding in a suitable machine.

A syrup may be made by adding the active compound to a concentrated aqueous solution of a sugar, for example sucrose to which may also be added any accessory ingredient(s). Such accessory ingredient(s) may include flavorings, suitable preservatives, an agent to retard crystallization of the sugar, and an agent to increase the solubility of any other ingredient, such as a polyhydric alcohol, for example glycerol or sorbitol.

Formulations suitable for parenteral administration conveniently comprise a sterile aqueous preparation of the active compound, which is preferably isotonic with the blood of the recipient.

Nasal spray formulations comprise purified aqueous solutions of the active compound with preservative agents and isotonic agents. Such formulations are preferably adjusted to a pH and isotonic state compatible with the nasal mucous membranes.

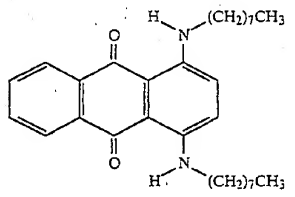
Formulations for rectal administration may be presented as a suppository with a suitable carrier such as cocoa butter, or hydrogenated fats or hydrogenated fatty carboxylic acids.

Opthalmic formulations are prepared by a similar method to the nasal spray, except that the pH and isotonic factors are preferably adjusted to match that of the eye.

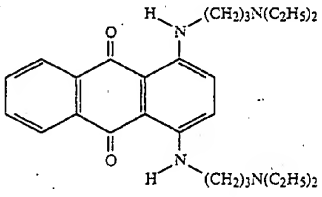
Topical formulations comprise the active compound dissolved or suspended in one or more media such as mineral oil, petroleum, polyhydroxy alcohols or other bases used for topical pharmaceutical formulations. The addition of other accessory ingredients, as described below, may be desirable.

In addition to the aforementioned ingredients, the formulations of this invention may further include one or more accessory ingredient(s) selected from diluents, buffers, flavoring agents, binders, disintegrants, surface active agents, thickeners, lubricants, preservatives (including antioxidants) and the like.

The following examples are provided to illustrate the present invention. Temperatures are given in degrees Celsius unless otherwise indicated. The following compounds were employed in the examples:



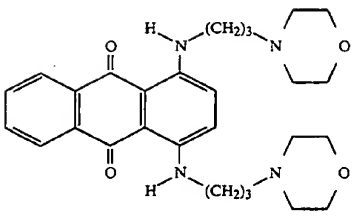
Compound 1a



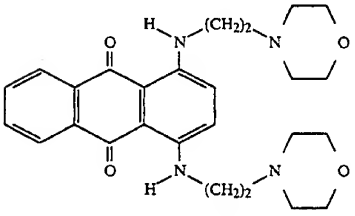
Compound 1b

-continued

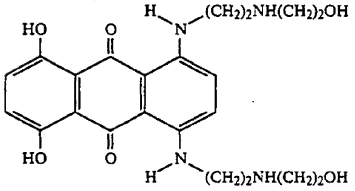
Compound 1c



Compound 1d



Mitoxantrone



EXAMPLE 1

The N-substituted diaminoanthraquinone, **1***b*, (1,4-bis-[[3'-(diethylamino)propyl]amino]anthraquinone), was identified as a PKC inhibitor in the micellar PKC screening assay and in platelets. This blue-colored compound had significantly greater activity in drug accumulation assays than other PKC inhibitors. Compound **1***c* (1,4-bis-[[3'(4-morpholino)propyl]amino]anthraquinone) had marginal PKC inhibitory activity in a preliminary screening assay. Examination of adherent cells treated with **1***b* or **1***c* revealed that treatment imparted a blue color to the monolayers. In Bligh-Dyer extracts of cells treated with **1***b* or **1***c*, the blue color was observed to quantitatively partition (>95%) with cellular phospholipids in the lower chloroform phase. Spectrophotometric analysis of **1***b* and 1c indicated they were excellent chromophores with absorbance maxima at 648 and 600 nm in chloroform, respectively, and extinction coefficients of approximately 20,000. At these wavelengths, there was minimal absorbance by cellular components contained in the chloroform phase. These properties of **1***b* and **1***c* were utilized to determine the time course of accumulation of these compounds in MDR MCF7 Adr 10 cells.

METHOD: Near confluent monolayers of MCF7 ADR 10 were washed 2 times with Dulbecco's Phosphate Buffered Saline (DPBS) and 1 ml of Iscove's Modified Dulbecco's Medium (IMDM) was added to each well. The monolayers were treated with 25 μM **1***b* or **1***c* and incubated at 37° C. After incubating the samples for the times indicated in FIG. 1, the monolayers were washed three times with DPBS and detached using 1 ml of Trypsin-EDTA. An aliquot of the cell suspension was counted using a Coulter Counter and a 0.8 ml aliquot was extracted in a Bligh-Dyer extraction (Bligh et at., *Can. J. Biochem.* 31:911-916 (1959)). The absorbance of the lower chloroform phase was measured at 648 nm and 600 nm using a spectrophotometer. The amount of **1***b* or **1***c* present in the extract was quantitated from a standard curve performed in cell extracts. The data is expressed as nmol anthraquinone/10^6 cells.

FIG. 1 shows the results of time course experiments which measured accumulation of **1***b* and **1***c*. Maximal accumulation of **1***b* and **1***c* was observed at about 2 hours.

EXAMPLE 2

The activities of **1***a*, **1***b*, **1***c* and **1***d* and mitoxantrone, an anthraquinone antineoplastic drug, were evaluated in vinblastine drug accumulation assays performed by the following method. 1,4-bis-(octylamino) anthraquinone (**1***a*), is a compound that caused PKC stimulation in screening assays. Mitoxantrone was identified as a PKC inhibitor in miceliar PKC assays. Compound **1***d* (1,4-bis-[[2-(4-morpholino)ethyl]amino]anthraquinone) was only evaluated in cellular studies.

METHOD: Washed cell monolayers were incubated in 1 ml of IMDM with the indicated diaminoanthraquinones or verapamil (a positive control) for 2 hours. [^{3}H] vinblastine ([^{3}H] VLB) was added to a final concentration of 100 nM and the cells were incubated for 2.5 hours. At this time, the cells were washed 3 times in ice cold PBS and lysed in buffer containing 0.1% Triton X-100. Net accumulation of [^{3}H] vinblastine was expressed as pmol/mg protein. In [^{3}H] VLB accumulation assays, basal accumulation of cells treated with vehicle alone were negative controls ([Drug]=0) and cells treated with verapamil are positive controls.

The activities of these compounds in vinblastine accumulation assays are summarized in FIGS. 2 and 3. FIG. 2 shows the results of vinblastine accumulation experiments in which the activities of **1***c* and verapamil are directly compared at concentrations ranging from 50 nM to 100 μM. The activities of **1***b* and 1,4-bis-[3'-(N-morpholino)ethylamino]anthraquinone (**1***d*) in vinblastine accumulation assays are shown in FIG. 3. These figures show that **1***b*, **1***c* and **1***d* increase vinblastine accumulation in MDR MCF7, and show that **1***c* is the most potent compound. In addition, **1***c* showed activity superior to verapamil at concentrations between 0.5 μM and 10 μM. A comparison of FIGS. 2 and 3 show that the lowest concentrations of each agent which cause a doubling of basal accumulation of vinblastine are 0.5 μM for **1***c*, 1 μM for verapamil, 2.5 μM for **1***d* and 25 μM for **1***b*. Compound **1***a* and mitoxantrone did not show significant activity in this assay.

EXAMPLE 3

The activity of the most potent compound, **1***c*, has been evaluated in vinblastine accumulation assays with intrinsically resistant renal cell carcinoma cell lines, A704, SKRC7 and UOK-39. These cell lines are models of intrinsic resistance. They express P-glycoprotein (demonstrated by immunocytochemistry using C219 monoclonal antibody to P-glyeoprotein). In MTT proliferation assays[1], the vinblastine IC50s for these cell lines are 10–600 fold higher than the vinblastine IC50 of sensitive MCF7. In contrast to the MCF7 Adr 10 cell line, a model of acquired MDR phenotype, the resistance in these cell lines has not been selected by exposure to increasing concentrations of natural product drugs. This in vitro data is consistent with the clinical observation that renal cell carcinoma is generally unre-

sponsive to chemotherapy. These tumors are considered intrinsically resistant (Chabner et. al., *Cancer* 54:2599–2608 (1984)).

[1] (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide). MTT, a yellow, water soluble tetrazolium dye, is reduced to an insoluble purple formazan by mitochondrial dehydrogenases in viable cells (Mosmann et at., *Immunochem. Meth.* 65:55–63 (1983)).

METHOD: Washed cell monolayers were incubated in 2 ml of IMDM with the indicated diaminoanthraquinones or verapamil for 2 hours. [^{3}H] vinblastine was added to a final concentration of 10 nM and the cells were incubated for 2.5 hours. At this time, the cells were washed 3 times in ice cold PBS and lysed in buffer containing 0.1% Triton X-100.

In FIGS. **4–6**, the net accumulation of [^{3}H] vinblastine is expressed as pmol [^{3}H] vinblastine/mg protein. These examples show that **1***c* increases vinblastine accumulation in cell lines which are models of intrinsic resistance. A comparison of the activities of **1***c* and verapamil (a positive control) at 10 μM, shows that **1***c* activity is comparable or superior to verapamil activity.

EXAMPLE 4

In 72 hour MTT proliferation assays using the following method, compounds **1***b*, **1***c* and **1***d* were assessed for inhibition of Vinca alkaloid and anthracycline drug resistance in MDR MCF7. Additionally, compounds **1***b* and **1***c* were assessed for potentiation of sensitivity to Vinca alkaloid and anthracycline drugs in the parental MCF7 WT cell line.

METHOD: 5000 cells per well were plated in 100 μl of IMDM containing 10% FCS and allowed to adhere overnight. The following day the cells were treated with N-substituted 1,4-diaminoanthraquinones and exposed to graded concentrations of antineoplastic agents. 50 μl of IMDM 10% FCS containing either vehicle or N-substituted 1,4-diaminoanthraquinones at 3 times the final tested concentration was added to cells at timed intervals. The plates were incubated for 2 hours at 37° C. to allow maximal cellular accumulation of N-substituted 1,4-diaminoanthraquinones. 40 μl aliquots of IMDM 10% FCS containing either vehicle or graded concentrations of antineoplastic drugs at 5 times the final concentration were added to treatment groups. Following this addition, 10 μl of IMDM 10% FCS containing either vehicle or N-substituted 1,4-diaminoanthraquinone at 5 times the final concentration was added to each group of wells. The cells were incubated for 72 hours after treatment. At this time the viable cells in each treatment group were estimated in a colorimetric assay by adding MTT and incubating the cells at 37° C. for 3–4 hours. The media in each well was aspirated and MTT formazan was solubilized in isopropyl alcohol acidified with 0.1 N HCl. The absorbance of the solubilized formazan was quantitated using a plate reader spectrophotometer. Control experiments showed that the absorbance of MTT was proportional to cell number. The concentrations of antineoplastic drugs which inhibited proliferation by 50% (IC50's) were estimated by calculating a percentage from the ratio: antineoplastic drug treated groups/control group absorbance and plotting the dose response curve on semilog paper. The IC50 was estimated from this curve. To assess the effects of inhibition of resistance by N-substituted 1,4-diaminoanthraquinones, the percentages were calculated relative to cell groups treated with resistance modifier alone and IC50's calculated as above. A reversal factor was calculated by dividing the IC50 to the antineoplastic drug alone (Control group) by the IC50 to antineoplastic drug in cells treated with resistance modifier.

TABLE 1

Reversal of Vincristine (VCR) Resistance in MCF7 Adr 10 by Compound 1c

[1c] μM	VCR IC_{50} (μM)	Reversal Factor
0	3.3 (±0.30)	1.0
0.1	1.4 (±0.50)	2.3
0.25	0.67 (±0.13)	5.0
0.5	0.32 (±0.07)	10.4
1.0	0.13 (±0.05)	24.8

The data shown in Table 1 is the average (± standard deviation) of 3 experiments.

TABLE 2

Reversal of Vinblastine (VLB) Resistance in MCF7 Adr 10 by Compound 1c

[1c] μM	VLB IC_{50} (μM)	Reversal Factor
0	0.79 (±0.12)	1.0
0.25	0.23 (±0.05)	3.5
0.1	0.37 (±0.01)	2.2
0.5	0.093 (±0.006)	8.5
1.0	0.087 (±0.011)	9.2

The data shown in Table 2 is the average (± standard deviation) of 3 experiments.

TABLE 3

Reversal of Adriamycin (ADR) Resistance in MCF7 Adr 10 by Compound 1c

[1c] μM	ADR IC_{50} (μM)	Reversal Factor
0	38 (±5)	1.0
0.5	19 (±8)	2.0
1.0	11 (±7)	3.3

The data shown in Table 3 is the average (± standard deviation) of 4 experiments.

TABLE 4

Reversal of Daunorubicin (DNR) Resistance in MCF7 Adr 10 by Compound 1c

[1c] μM	DNR IC_{50} (μM)	Reversal Factor
0	6.7 (±1.0)	1.0
0.5	3.5 (±0.3)	1.9
1.0	2.1 (±0.3)	3.2

The data shown in Table 4 is the average (± standard deviation) of 3 experiments.

TABLE 5

Potentiation of Vincristine (VCR) Sensitivity in MCF7 WT by Compound 1c

[1c] μM	VCR IC_{50}(nM)	Reversal Factor
0	1.9 (±0.4)	1.0
1.0	1.0 (±0.2)	1.9

The data shown in Table 5 is the average (± standard deviation) of 5 experiments.

Comparisons of activities of **1***b*, **1***c* and **1***d* as inhibitors of drug resistance indicated that **1***c* was the most potent inhibitor of the MDR phenotype in the MCF7 Adr 10 cell line. In the inhibition concentration (IC) curves shown in FIGS. **7–10**, the inhibition of drug resistance to vincristine (VCR), vinblastine (VLB), adriamycin (ADR), and daunorubicin (DNR) in MDR MCF7 by 1c in representative experiments is shown. In each case, the IC50 concentration of the respective antineoplastic drug was reduced as a function of increasing **1***c* concentration. Tables 1–4 relating to FIGS. **7–10**, respectively, summarize the results of repeated experiments as an average (± standard deviation) and a reversal factor. In FIG. **11** and Table 5, potentiation of vincristine sensitivity in MCF7 WT is shown. The concentrations of **1***c* in these experiments were not cytotoxic. In the presence of **1***c* alone, the average cell growth was greater than

86% (n=14) of the vehicle control at the test concentrations. In the Paired Student's t-test, the differences in the antineoplastic drug IC50s for control ([1c]=0) and 1c treated cells are significant ($P<0.05$).

EXAMPLE 5

The effects of compound 1c on phosphorylation of the P-glycoprotein and the MARCKS protein (myristoylated alanine-rich C kinase substrate) was studied in intact cells labeled with [^{32}P] orthophosphate. The MARCKS protein (also known as the 80 to 87 kD protein) is observed to be rapidly phosphorylated subsequent to PKC activation by phorbol esters, cell permeant diacylglycerols, growth factor and hormonal stimulation in a variety of cell types from several species. MARCKS displays calcium-calmodulin binding and filamentous actin crosslinking activities which are regulated by PKC mediated phosphorylation (Hartwig et al., *Nature* 356:618–622 (1992)). In these experiments, phosphorylation of MARCKS was used as a marker of cellular PKC activity. The effects of 1c on phosphorylation states of the P-glycoprotein and MARCKS protein were studied to test the possibility that this compound may be inhibiting PKC in cells; due to metabolism to an active PKC inhibitor or by achieving inhibitory concentrations in the microenvironment of the P-glycoprotein.

METHOD: A monoclonal antibody to the P-glycoprotein, C219, was used to immunoprecipitate the P-glycoprotein and a polyclonal rabbit antibody was used to immunoprecipitate the MARCKS protein from extracts of cells labeled with [^{32}P]. MARCKS was immunoprecipitated after preclearing the aliquot with normal rabbit IgG. MCF7 Adr 10 cells were labeled with [^{32}P] orthophosphate for two hours. At timed intervals, cells were treated with 1c and incubated for 10 minutes to two hours. All samples were labeled for a total of 4 hours at which time the samples were washed and lysed in an extraction buffer containing detergents, protease inhibitors and phosphatase inhibitors. The samples were matched by TCA precipitable counts. P-glycoprotein and MARCKS were immunoprecipitated from aliquots of the same sample containing equivalent radioactivity.

The summary of densitometric studies of a representative time course experiment with 1c is shown in FIG. 12. Autoradiographs of this experiment shown in FIGS. 13 and 14 show that 1c increases both P-glycoprotein and MARCKS phosphorylation, 6.6-fold and 3-fold, respectively (FIG. 12). There is a good correlation between the time course of 1c accumulation measured spectrophotometrically from the organic phase of Bligh-Dyer extracts (FIG. 1) and the time course of P-glycoprotein and MARCKS phosphorylation (FIG. 12). These results suggest that 1c is not inhibiting PKC in intact cells. Previously, phorbol myristate acetate, an activator of PKC, and verapamil and trifluoperazine, resistance modifiers which bind to the P-glycoprotein and non-specifically inhibit PKC in vitro, have been found to stimulate phosphorylation of the P-glycoprotein at serine residues on different sites of the P-glycoprotein (Hamada et al., *Cancer Res.* 47:2860–2865 (1987)).

Compound 1c was evaluated for inhibitory activity against a variety of cellular kinases including casein kinase 2, the catalytic subunits of protein kinase A and calmodulin kinase; but 1c did not inhibit these kinases. One possible explanation for stimulation of phosphorylation of P-glycoprotein and MARCKS is that 1c may activate PKC or other cellular kinases by signal transduction pathways that have not yet been identified to phosphorylate these proteins. An alternative explanation for stimulation of phosphorylation of P-glycoprotein and MARCKS is that 1c inhibits protein phosphatases. Analysis of overall phosphoprotein changes in cell extracts indicates that treatment with 1c did not significantly alter phosphorylation of other phosphoproteins.

EXAMPLE 6

The effects of 1c on specific binding of [^{3}H] VLB to membrane vesicles from sensitive and MDR MCF7 have been studied using the method described below. These studies indicate that 1c inhibits vinblastine binding to the P-glycoprotein.

METHOD: Membrane vesicles from sensitive and MDR MCF7 were prepared by nitrogen cavitation and isolated by centrifugation in discontinuous sucrose gradients. SDS-PAGE analysis of membrane vesicles from sensitive and resistant MCF7 cells shows a 160 kD protein which is present in membrane vesicles from resistant cells and absent in MCF7 wt cells. This band can be immunoprecipitated by C219 antibody to P-glycoprotein indicating that membrane vesicles from MDR cells contain the P-glycoprotein and membrane vesicles from MCF7 WT do not. In the presence of 100 nM [^{3}H] VLB and ATP, total and nonspecific binding of [^{3}H] VLB (experimentally defined as binding in the presence of a 100 μM unlabeled VLB) was measured. Association of [^{3}H] VLB with membrane vesicles of MCF7 Adr 10 in a rapid filtration assay was: (1) maximal by 15 min. at room temperature; (2) inhibited by addition of unlabeled VLB; and (3) enhanced, approximately 2–3 fold, by addition of ATP. In contrast, association of [^{3}H] VLB to membrane vesicles of MCF7 WT was approximately 15 % of MCF7 Adr 10 and addition of ATP did not significantly enhance association of [^{3}H] VLB with MCF7 WT vesicles.

Previous studies have shown that membrane vesicles from MDR cells show specific and saturable binding of Vinca antineoplastic drugs (Hamada et al., *J. Biol. Chem.* 263:1454–1458, 1988; Cornwell et at., *J. Biol. Chem.* 261: 7921–7928 (1986); and Cornwell et al., *Proc. Natl. Acad. Sci., USA* 83:3847–3850 (1986)). Thus, the differences in association of [^{3}H] VLB with membrane vesicles of MCF7 WT and MCF7 Adr 10 are probably related to binding of [^{3}H] VLB by the P-glycoprotein in membrane vesicles of the MDR cells.

Specific binding of [^{3}H] VLB to MCF7 Adr 10 membrane vesicles was 27.4 pmol/mg protein. It was determined by subtracting non-specific binding from total binding. In the presence of 1c at 5, 10, and 25 μM, specific binding was decreased by 29, 42 and 69%, respectively. 1c had no effect on non-specific binding of [^{3}H] VLB to MCF7 Adr 10 vesicles, or the small specific binding of [^{3}H] VLB to MCF7 WT membrane vesicles. These observations suggest that modulation of the MDR phenotype by 1c may be a result of inhibition of binding of natural product drugs by P-glycoprotein. Thus, both binding and phosphorylation of P-glycoprotein may be important in modulation of the MDR phenotype by 1c.

EXAMPLE 7

Myelosuppression is often a dose limiting side effect of chemotherapeutic agents. Generally, most anticancer

drugs are more or equally toxic to bone marrow granulocyte-macrophage progenitors (CFU-GM) than to cancer cells (Fine et at., *J. Clin. Oncol.* 5:489–493 (1986)). The toxicities to human granulocyte-macrophage progenitors (CFU-GM) of **1***b*, **1***c*, and VLB was assessed by measuring inhibition of CFU-GM proliferation in cultures continuously exposed to each agent alone and compared to the toxicities to sensitive and resistant MCF7 in tumor cell clonogenic assays.

METHOD: The CFLI-GM assay measures clonal proliferation of granulocyte/macrophage precursors in semisolid agar cultures. The progeny of the granulocyte/macrophage precursors remain in proximity of the progenitor cells and form colonies. Colonies which contain greater than 40 cells after 10–12 days of culture are counted using an inverted phase contrast microscope. The tumor cell clonogenic assays measure clonal proliferation of tumor cells. In this assay, 300–500 cells are plated in 10 cm^2 wells, allowed to adhere overnight, and are treated the following day. Tumor cell colonies were counted after 10–12 days of culture. The times of exposure in these experiments are similar; however, the concentrations of FCS are 20% in the CFU-GM assay and 10% in the tumor cell clonogenic assays. The IC50's are calculated as described above for the MTT proliferation assays. (See Example 4).

In Table 6, the IC50s for **1***c*, **1***b* and VLB in MCF7 WT and MCF7 ADR 10 cells clonogenic assays are compared to the IC50s in the CFU-GM bone marrow assay. To facilitate this comparison, a CFLI-GM toxicity index was calculated by dividing the CFU-GM IC50 by the respective tumor cell clonogenic IC50's in sensitive and resistant cells. A comparison of the IC50s in clonogenie assays of sensitive MCF7 WT and MDR MCF7 Adr 10 to the natural product drugs adriamycin (ADR), vinblastine (VLB) and vincristine (VCR) in Table 6 indicate that much higher concentrations of these drugs were required for cytotoxicity in the MCF7 Adr 10 line which exhibit the MDR phenotype. A comparison of the IC50s to vinblastine for CFU-GM, sensitive and MDR MCF7 indicates that vinblastine is approximately twice as toxic to CFU-GM as it is to the drug sensitive WT cancer cells and 1000 times more toxic to CFU-GM than to the MDR cells. The CFU-GM toxicity indices shown for vinblastine are less than 1, indicating that vinblastine is more toxic to CFU-GM than to sensitive and MDR MCF7. MCF7 WT are more sensitive to cytotoxicity of **1***b* than MDR MCF7 and both tumor lines were 2 to 3.5 fold more sensitive to **1***b* cytotoxicity than CFU-GM as indicated by CFU-GM toxicity indices which are greater than 1. In contrast, MDR MCF7 are twice as sensitive to **1***c* cytotoxicity as MCF7 WT and CFU-GM are approximately 10 to 20 times less sensitive to **1***c* cytotoxicity than drug sensitive (WT) and MDR cancer cell lines. These observations suggest that **1***c* has a large therapeutic index for bone marrow toxicity versus tumor cell toxicity, especially for MDR cells.

TABLE 6

A Comparison of Human Cancer Cell And CFU-GM Toxicity

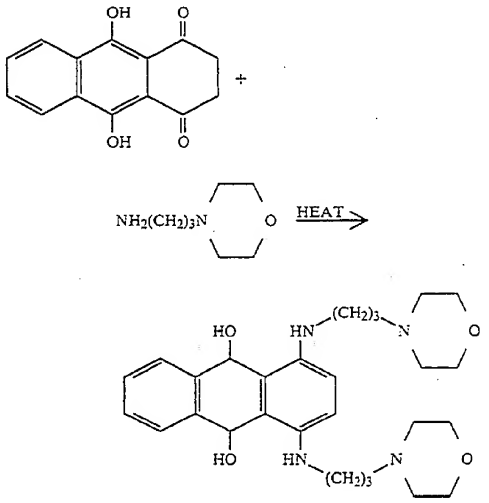
DRUG	MCF7 WT	MCF7 ADR 10	CFU-GM
	Continuous Exposure IC_{50}		
1b	0.4 μM	0.7 μM	1.4 μM
1c	0.8 μM	0.4 μM	7.8 μM
VLB	0.59 nM	0.21 μM	0.28 nM
ADR	5.6 nM	3.4 μM	—
VCR	0.4 nM	0.97 μM	—

TABLE 6-continued

A Comparison of Human Cancer Cell And CFU-GM Toxicity

DRUG	MCF7 WT	MCF7 ADR 10	CFU-GM
	CFU-GM IC_{50} Tumor Cell Clonogenic IC50 (CFU-GM Toxicity Index)		
1b	3.5	2.0	
1c	9.8	19.5	
VLB	0.47	0.001	

EXAMPLE 8



A mixture of leucoquinazarin (2,3-dihydro-9,10-dihydroxy-1,4-anthracenedione, 24.2 g, 0.1 tool) and 4-(3-aminopropyl)morpholine, 57.6 g, 0.4 mol) was heated at 50° C. under an atmosphere of N_2 for 90 min. The mixture was cooled and allowed to stand overnight. The oily residue was taken up in 600 ml of CH_3OH, and air was bubbled through the mixture at 50° C. for 3 h. After removal of the solvent under reduced pressure, the residue was purified by column chromatography on silica gel (1.5 kg). Elution with 5 % CH_2Cl_2 in hexane gave 13.5 g pure product. A second fraction collected (20.3 g) contained trace impurity. The first fraction was recrystallized from EtOH to give 11.3 g of pure sample: mp 134°–136° C., ^1H NMR ($CDCl_3$, 250 MHz) δ 1.93 (m, 4), 2.49 (m, 12), 3.50 (m, 4), 3.74 (m, 8), 7.29 (s, 2), 7.69 (m, 2), 8.34 (2, m).

Anal. calc'd for $C_{28}H_{36}N_4O_4$ (492.61): C, 68.27; H, 7.37; N, 11.38. Found: C, 68.35; H, 7.53; N, 11.28.

In summary, aminoanthraquinone resistance modifiers have been identified which have the structural and functional features of a generally described MDR phenotype pharmacophore. Specifically, they are lipophilic compounds with planar aromatic domains and a basic nitrogen atom. The present data indicate that inhibition of the drug accumulation defect of MDR cells by aminoanthraquinones is influenced by the following structural characteristics. First, a nitrogen is required in the alkyl side chain[2] for activity. Compound **1***a* which lacks a nitrogen in the alkyl side chain is inactive. Second, the presence of a hydrophilic group, such as a heterocyclic ring, on the alkyl side chain is associated with greater

activity than hydrophobic groups, such as branched alkyl chains. Compound **1c**, with a heterocyclic morpholino functional group has greater activity than **1b** with branched ethyl chains. Third, the position of the alkyl side chain nitrogen relative to the amino anthraquinone ring is important. Compound **1c** with three carbon atoms between the nitrogen atoms[3] is a more potent MDR phenotype inhibitor than **1d** which has two carbon atoms between the nitrogen atoms.[4]Finally, hydroxy substitutions at the 9, 10 positions of anthraquinone ring and on the ethyl side chains of mitoxantrone are associated with a significant loss of activity.

[2] i.e., M in Formulae I and II.
[3] i.e., x=3
[4] i.e., x=2

The activity of these compounds in vinblastine accumulation assays does not correlate with in vitro inhibition of serine threonine kinases. Mitoxantrone shows greater inhibitory activity in in vitro assays of PKC and casein kinase 2 than **1b** or **1c**, but does not increase drug accumulation. However, unlike **1c**, **1a**, **1b** and **1d**, mitoxantrone does not partition into the lower chloroform phase. It partitions into the upper methanol:water phase indicating that it is much less lipophilic. In contrast, **1c**, the most potent inhibitor of the MDR phenotype, shows the greatest activity in drug accumulation assays but does not show significant activity in in vitro assays of serine threonine kinases.

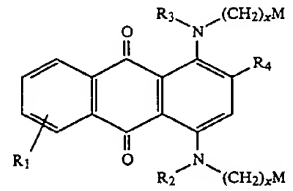
In contrast to verapamil, phenothiazines, thioxanthenes or reserpine analogs which inhibit the MDR phenotype at between 2–10 μM (Ford et at., *Cancer Res.* 50:1748–1756 (1990); Zamora et al., *Mol. Pharmacol.* 33:454–462 (1988); Pearce et al., *Proc. Natl. Acad. Sci., USA,* 86:5128–5132 (1989); and Ford et at., *Pharmacol. Rev.* 42:155–198 (1990)), compound **1c** inhibits the MDR phenotype at concentrations between 100 nM and 1 μM and enhances toxicity of vincristine in drug sensitive cells. The antiproliferative effects of compound **1c** alone in clonal proliferation assays are approximately 10 and 20 times greater, respectively, in sensitive and MDR cancer cells than in human CFU-GM. These studies suggest that **1c** has a large therapeutic index for bone marrow toxicity versus cancer cell toxicity.

We claim:

1. A method of reducing drug-resistance in a subject having a drug-resistant cancer, said method comprising:

administering to the subject a drug-resistance reducing amount of a potentiating agent, wherein said potentiating agent is a compound selected from the group consisting of a compound of Formula (I), a compound of Formula (II), a pharmaceutically acceptable salt thereof and combinations thereof, wherein

Formula I



and

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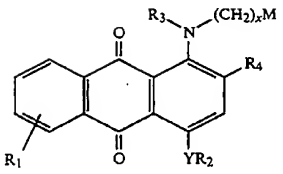
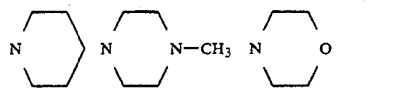
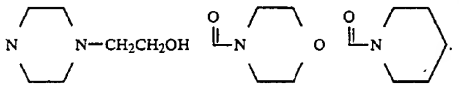
Formula II



wherein:

R_1 is H or an alkyl chain with 1-7 carbon atoms;
R_2 is H or an alkyl chain with 1-7 carbon atoms;
R_3 is H or an alkyl chain with 1-7 carbon atoms;
R_4 is H or an alkyl chain with 1-7 carbon atoms;
x is an integer ranging from 3 to 12;
Y is O or NH; and
M is selected from the group of moieties consisting of:

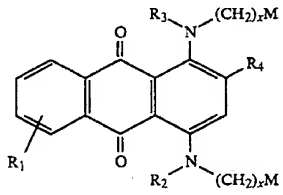




2. The method of claim **1**, wherein the potentiating agents are compounds according to Formula I, or a pharmaceutically acceptable salt thereof, or combinations thereof, and wherein R_1, R_2, R_3, and R_4 are H.

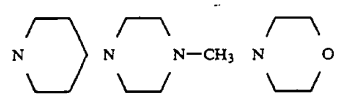
3. A pharmaceutical composition for the potentiation of the effect of anticancer drugs, said composition comprising a potentiating agent together with a pharmaceutically acceptable carrier or diluent, and an antineoplastic drug, wherein said potentiating agent is a compound selected from the group consisting of a compound of Formula (I), or a pharmaceutically acceptable salt thereof or combinations thereof, wherein

Formula I



wherein:

R_1, R_2, R_3, and R_4 are H;
x is an integer ranging from 3 to 12;
Y is O or NH; and
M is selected from the group consisting of:



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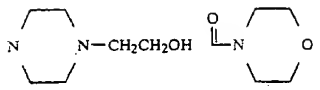
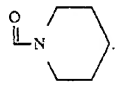
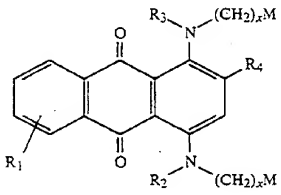




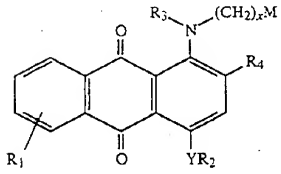
4. A method for sensitizing multidrug resistant tumor cells to an antineoplastic drug, said method comprising:

administering to multidrug resistant tumor cells an effective sensitizing amount of a potentiating agent and an effective anti-tumor amount of an antineoplastic drug, wherein said potentiating agent is a compound selected from the group consisting of a compound of Formula (I) , a compound of Formula (II), a pharmaceutically acceptable salt thereof and combinations thereof, wherein



Formula I

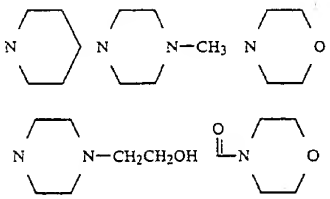
and



Formula II

wherein:

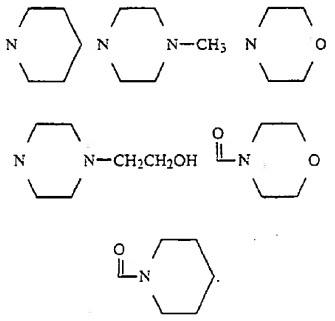
R_1 is H or an alkyl chain with 1–7 carbon atoms;
R_2 is H or an alkyl chain with 1–7 carbon atoms;
R_3 is H or an alkyl chain with 1–7 carbon atoms;
R_4 is H or an alkyl chain with 1–7 carbon atoms;
x is an integer ranging from 3 to 12;
Y is O or NH; and
M is selected from the group consisting of:



$N(CH_2CH_2OH)_2$ $NHCH_2CH_2OH$.

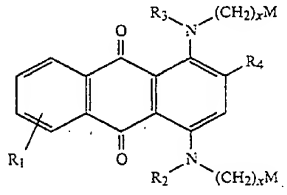
5. The method of claim 4, wherein the antineoplastic drug is selected from the group consisting of Vinca alkaloids, anthracyclines, antibiotics, epipodophlotoxins, topoisomerase I and II inhibitors, taxol, taxotere, and taxol derivatives.

6. The method of claim 4, wherein M is selected from the group consisting of:



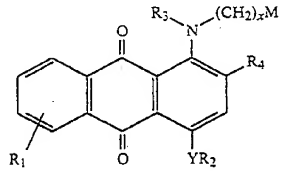
7. The method of claim 6, wherein the potentiating agents are compounds according to Formula I or a pharmaceutically acceptable salt thereof or combinations thereof, wherein R_1, R_2, R_3, and R_4 are H.

8. A pharmaceutical formulation comprising an effective antitumor amount of an antineoplastic agent and an effective sensitizing amount of a potentiating agent, wherein said potentiating agent sensitizes multidrug resistant tumor cells to said antitumor agent and wherein said potentiating agent is a compound selected from the group consisting of a compound of Formula (I), a compound of Formula (II), a pharmaceutically acceptable salt thereof and combinations thereof, wherein



Formula I

and



Formula II

wherein:

R_1 is H or an alkyl chain with 1–7 carbon atoms;
R_2 is H or an alkyl chain with 1–7 carbon atoms;
R_3 is H or an alkyl chain with 1–7 carbon atoms;
R_4 is H or an alkyl chain with 1–7 carbon atoms;
x is an integer ranging from 3 to 12;
Y is O or NH; and
M is selected from the group consisting of:

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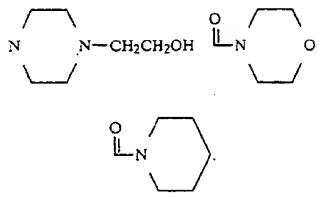
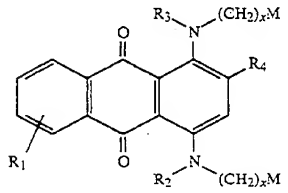


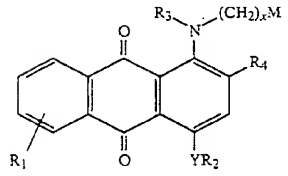
4. A method for sensitizing multidrug resistant tumor cells to an antineoplastic drug, said method comprising:

administering to multidrug resistant tumor cells an effective sensitizing amount of a potentiating agent and an effective anti-tumor amount of an antineoplastic drug, wherein said potentiating agent is a compound selected from the group consisting of a compound of Formula (I) , a compound of Formula (II), a pharmaceutically acceptable salt thereof and combinations thereof, wherein



Formula I

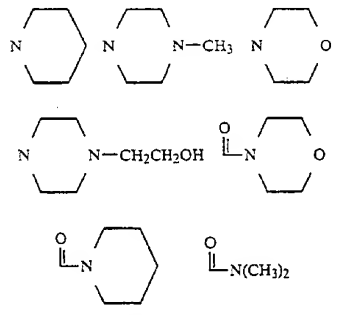
and



Formula II

wherein:

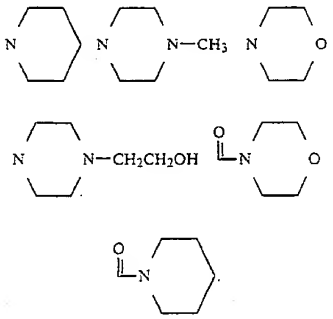
R_1 is H or an alkyl chain with 1–7 carbon atoms;
R_2 is H or an alkyl chain with 1–7 carbon atoms;
R_3 is H or an alkyl chain with 1–7 carbon atoms;
R_4 is H or an alkyl chain with 1–7 carbon atoms;
x is an integer ranging from 3 to 12;
Y is O or NH; and
M is selected from the group consisting of:



$N(CH_2CH_2OH)_2$ $NHCH_2CH_2OH$.

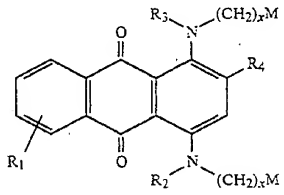
5. The method of claim 4, wherein the antineoplastic drug is selected from the group consisting of Vinca alkaloids, anthracyclines, antibiotics, epipodophlotoxins, topoisomerase I and II inhibitors, taxol, taxotere, and taxol derivatives.

6. The method of claim 4, wherein M is selected from the group consisting of:



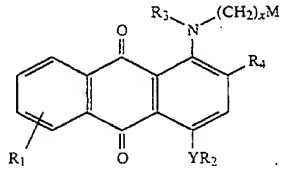
7. The method of claim 6, wherein the potentiating agents are compounds according to Formula I or a pharmaceutically acceptable salt thereof or combinations thereof, wherein R_1, R_2, R_3, and R_4 are H.

8. A pharmaceutical formulation comprising an effective antitumor amount of an antineoplastic agent and an effective sensitizing amount of a potentiating agent, wherein said potentiating agent sensitizes multidrug resistant tumor cells to said antitumor agent and wherein said potentiating agent is a compound selected from the group consisting of a compound of Formula (I), a compound of Formula (II), a pharmaceutically acceptable salt thereof and combinations thereof, wherein



Formula I

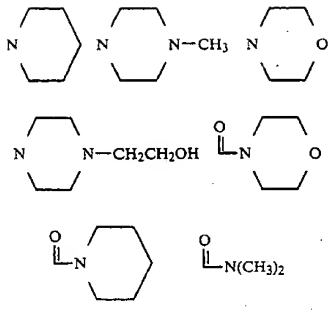
and



Formula II

wherein:

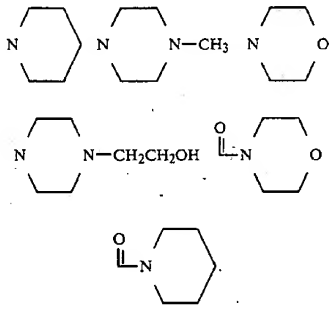
R_1 is H or an alkyl chain with 1–7 carbon atoms;
R_2 is H or an alkyl chain with 1–7 carbon atoms;
R_3 is H or an alkyl chain with 1–7 carbon atoms;
R_4 is H or an alkyl chain with 1–7 carbon atoms;
x is an integer ranging from 3 to 12;
Y is O or NH; and
M is selected from the group consisting of:



$N(CH_2CH_2OH)_2$ $NHCH_2CH_2OH$.

9. The formulation of claim 8, wherein the antineoplastic drug is selected from the group consisting of Vinca alkaloids, anthracyclines, antibiotics, epipodophlotoxins, topoisomerase I and II inhibitors, taxol, taxotere, and taxol derivatives.

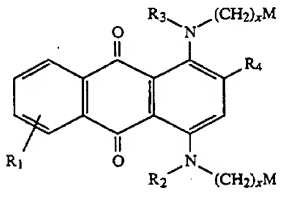
10. The formulation of claim 8, wherein M is selected from the group consisting of:



11. The formulation of claim 8, wherein the potentiating agents are compounds according to Formula I or a pharmaceutically acceptable salt thereof or combinations thereof, and wherein R_1, R_2, R_3, and R_4 are H.

12. A method of selectively inhibiting the growth of tumor cells in a subject in need of such treatment, said method comprising:

administering to said subject an effective amount of an anticancer drug independently or in combination with a potentiating agent, wherein said potentiating agent is a compound selected from the group consisting of a compound of Formula (I), a compound of Formula (II), a pharmaceutically acceptable salt thereof and combinations thereof, wherein



Formula I

and

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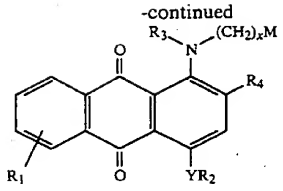


Formula II

wherein:

R_1 is H or an alkyl chain with 1–7 carbon atoms;

R_2 is H or an alkyl chain with 1–7 carbon atoms;

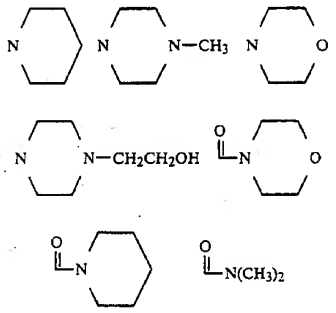
R_3 is H or an alkyl chain with 1–7 carbon atoms;

R_4 is H or an alkyl chain with 1–7 carbon atoms;

x is an integer ranging from 3 to 12;

Y is O or NH; and

M is selected from the group consisting of:

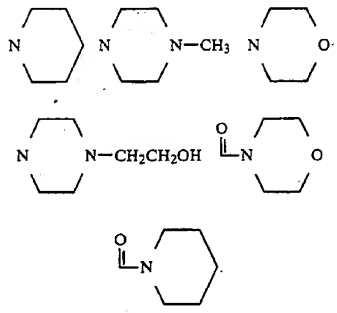


$N(CH_2CH_2OH)_2$ $NHCH_2CH_2OH$.

13. The method of claim 12, wherein the antineoplastic drug is selected from the group consisting of Vinca alkaloids, anthracyclines, antibiotics, epipodophlotoxins, topoisomerase I and II inhibitors, taxol, taxotere, and taxol derivatives.

14. The method of claim 12, wherein the amount of the potentiating agent administered ranges from about 1 to about 400 mg/kg/day in a single or divided dose.

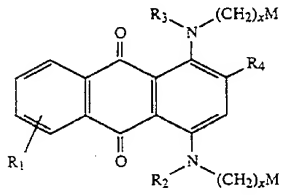
15. The method of claim 12, wherein M is selected from the group consisting of:



16. The method of claim 12, wherein the potentiating agents are compounds according to Formula I or a pharmaceutically acceptable salt thereof or combinations thereof, and wherein R_1, R_2, R_3, and R_4 are H.

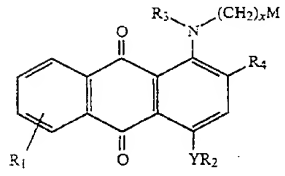
17. A method for sensitizing tumor cells to an antineoplastic drug, said method comprising:

administering to said tumor cells an effective sensitizing amount of a potentiating agent and an effective anti-tumor amount of said antineoplastic drug, wherein said potentiating agent is a compound selected from the group consisting of a compound of Formula (I), a compound of Formula (II), a pharmaceutically acceptable salt thereof and combinations thereof, wherein



Formula I

and



Formula II

wherein:

R_1 is H or an alkyl chain with 1–7 carbon atoms;

R_2 is H or an alkyl chain with 1–7 carbon atoms;

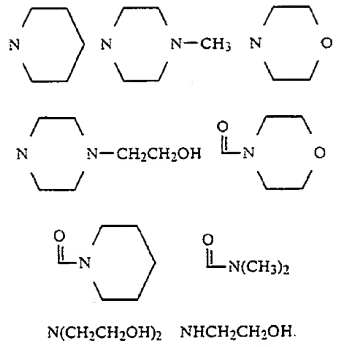
R_3 is H or an alkyl chain with 1–7 carbon atoms;

R_4 is H or an alkyl chain with 1–7 carbon atoms;

x is an integer ranging from 3 to 12;

Y is O or NH; and

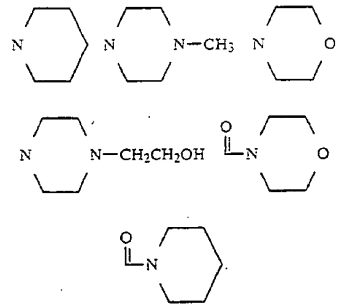
M is selected from the group consisting of:



$N(CH_2CH_2OH)_2$ $NHCH_2CH_2OH$.

18. The method of claim 17, wherein the antineoplastic drug is a Vinca alkaloid.

19. The method of claim 17, wherein M is selected from the group consisting of:



20. The method of claim 19, wherein the potentiating agents are compounds according to Formula I or a pharmaceutically acceptable salt thereof or combinations thereof; and wherein R_1, R_2, R_3, and R_4 are H.

* * * * *

Any further treatment for this patient with pancreatic cancer?

From PRIMARY CARE & CANCER, Vol 17, No 7 (July/August 1997)

Two months ago, my patient was diagnosed with pancreatic cancer and liver metastasis. He has experienced approximately a 50-lb weight loss in 3 to 4 months, and he is anemic. He is currently on gemcitabine. I will have him undergo a repeat CT scan to determine treatment progress. The patient has a stent in the common duct and occasional epigastric pain that is relieved by acetaminophen with codeine (30 mg). Nausea is relieved with lorazepam, 2 mg sublingual. What further treatment would you recommend for this patient?

A family practitioner in Escondido, California

More clinical information is needed on this patient with metastatic pancreatic cancer to make treatment recommendations. For example, what are his current medical condition and performance status? How is he tolerating gemcitabine, and is there any evidence of clinical response or benefit?

There are few options for treating metastatic pancreatic cancer. The majority of patients experience rapid clinical deterioration and a short survival measured in months. The antitumor response to available chemotherapy agents is modest. The well-known resistance of pancreatic cancer to chemotherapy prompts many patients to opt only for best supportive medical care without even a trial of chemotherapy.

Palliation of metastatic pancreatic cancer is a complex and demanding undertaking. Pain from the disease, perhaps the most difficult cancer-related pain to treat, is complicated by coexistent anorexia, fatigue, and clinical depression. However, recent studies evaluating the management of cancer pain have indicated that, with aggressive use of medications, most patients with pancreatic cancer can achieve satisfactory control of pain.

The armamentarium of pain medications has now increased to include short-, intermediate, and long-acting preparations of narcotic analgesics, transdermal delivery of anesthetics, continuous subcutaneous or parenteral delivery of narcotics via ambulatory infusion pumps, and adjuvant agents including nonsteroidal anti-inflammatory drugs and tricyclic antidepressants.

For pain refractory to a trial of serial analgesics, celiac nerve block is often effective and safe, which may augment the use of narcotics. Because of the length, complexity, and expense of treatment, multifield beam radiotherapy is not generally considered for the palliation of patients with pain from metastatic pancreatic cancer, except in cases of locally unresectable disease. In these cases, significant palliation of pain is occasionally achieved.

Depression and fatigue need to be treated as aggressively as pain, and use of antidepressants and stimulants (particularly if patients are heavily medicated with opiates) should be routinely considered. The use of megestrol acetate may also benefit some patients as an appetite stimulant.

Trials of single-agent or combination chemotherapy have been consummately disappointing in pancreatic cancer. Promising early results published in phase II trials have often failed to indicate an enhanced tumor response in phase III trials. Conventional single agents such as fluorouracil, mitomycin (Mutamycin), and doxorubicin have limited antitumor response and little if any palliative benefit, and combination therapy with these agents has increased treatment toxicity without any convincing effect on treatment efficacy. Biomodula-tion of fluorouracil using drugs such as folinic acid or interferon has also failed to improve antitumor response.

The identification of gemcitabine (Gemzar) as an active agent has represented a modest advance in the treatment of pancreatic cancer, as borne out in a recent national trial that randomized patients with unresectable or metastatic pancreatic cancer to receive fluorouracil or gemcitabine. The primary study endpoint was clinical response benefit measured as improvement in clinical parameters such as performance status and pain control. Antitumor response and survival were secondary endpoints.

Gemcitabine appeared to be superior, with significant clinical improvement seen in roughly one-quarter of patients treated, compared with little or no clinical improvement with fluorouracil. Patients treated with gemcitabine had a greater median and 1-year survival, and the major antitumor response, although modest, was superior for gemcitabine compared with fluorouracil. The results of this trial led to the recent FDA approval of gemcitabine for un-resectable or metastatic pancreatic cancer.

If this patient has disease progression on gemcitabine, the next consideration for treatment should be enrollment in an investigational drug trial, given the limited efficacy of conventional chemotherapy agents in metastatic pancreatic cancer. Irinotecan (Camptosar), which was recently approved for use in colorectal cancer, appears to have some antitumor activity in pancreatic cancer; further study of this drug as a single agent and in combination therapy is planned in trials to be conducted in the US. Studies of the taxanes in this setting are also ongoing, although these agents appear to have limited antitumor activity in treating metastatic pancreatic cancer.

Single-agent fluorouracil, mitomycin, or doxorubicin have limited antitumor activity even in chemotherapy-naive patients, and can be considered as conventional second-line therapies. Combination therapy with these agents is difficult to justify, given randomized trial data that have not shown a significant response or survival benefit.

Finally, in light of the noncurative nature of chemotherapy, best supportive care without further chemotherapy is also an option. Ongoing laboratory investigation in pancreatic cancer will hopefully reveal new biochemical targets for potential chemotherapeutic intervention. Such biochemical targets include growth factor receptors and their signal transduction pathways. Of particular interest is the ras oncogene, which is mutated in more than 90% of pancreatic tumors. Potential anti-ras drugs are already far along in preclinical development. Tumor angiogenesis factors, factors involved in tumor invasion and metastasis, and cell-cycle regulatory factors are also under active investigation.

DAVID H. IISON, MD, PhD
Assistant Attending Physician
Gastrointestinal Oncology Service
Department of Medicine
Memorial Sloan-Kettering Cancer Center
New York, New York
